JOURNAL OF AGRICULTURAL RESEARCH

VOL. XXVI WASHINGTON, D. C., NOVEMBER 24, 1923 NO. 8

COMPOUNDS DEVELOPED IN RANCID FATS, WITH OBSERVATIONS ON THE MECHANISM OF THEIR FORMATION [1]

By WILMER C. POWICK

Biochemist, Biochemic Division, Bureau of Animal Industry, United States Department of Agriculture

INTRODUCTION

The term "rancidity" is used to denote a certain type of change or spoilage in fats and oils that is characterized by a distinctive disagreeable odor and taste. While the term is sometimes used to describe the changes wrought by bacteria and fungi in such products as butter, it will be limited in this paper to spoilage that may occur in purified fats under conditions precluding the action of biological agents. It will be distinguished further from the condition of acidity, which is not necessarily associated with an objectionable odor and taste and which is developed by a fairly well understood mechanism.

Previous investigators (*5; 10; 20; 24, p. 52; 32; 36*) studying the chemistry of rancidity have concluded that this condition results through the oxidation of fats by atmospheric oxygen in the presence of light or of certain metals as catalysts. While the mechanism of this action is far from being clear, some of the products formed during the development of rancidity have been characterized.

Thus, azelaic acid (*27*) and most of the saturated acids and aldehydes, from acetic up to nonylic (*37*), have been recovered from certain specimens of rancid fats and adequately identified, and at least one investigator (*37*) has held heptylic aldehyde to be responsible for the rancid odor. The presence of acrolein (*5*) and of glyceric aldehyde (*40*) in rancid fats has also been a matter of occasional speculation, but no satisfactory evidence on this point has been advanced. While the data available do not relate to a sufficient variety of samples, perhaps, to justify the conclusion that any one of these compounds is a constituent of every rancid fat, it is a matter of common knowledge that qualitative tests indicate that the aldehyde group is regularly present, as is also the peroxid group. Determinations of acetyl values (*5, 13*), also, have indicated the gradual formation of hydroxy compounds during the development of rancidity, while other evidence (*14*) points to the formation of polymerization products. If we add to the foregoing the fact that there seems to be little room for doubt that in the development of rancidity the point of

[1] Accepted for publication June 25, 1923. This is the second of a series of "Studies on Rancidity." (*10*).[2]

[2] Reference is made by number (italic) to "Literature cited," p. 360–362.

Journal of Agricultural Research, Washington, D. C.
ah t

Vol. XXVI, No. 8
Nov. 24, 1923
Key No. A-56

60879—24——1

initial attack by the atmospheric oxygen is the oleic acid radical (*5*, *36*) we will have summarized briefly the present status of our knowledge concerning the chemistry of rancidity.

While much of the incentive for more detailed study of the chemistry of rancidity was removed with the discovery that rancidity is caused by a process of oxidation and can be prevented by exclusion of light and air, such a study is still of interest from the point of view of food control, both in connection with the problem of detecting rancidity in fats and in its bearing upon the question of the wholesomeness of rancid fats when used as food.

Logically, the term "rancid" is descriptive of odor and taste, and rancidity as such is properly detected and estimated by means of the sense perceptions; yet in practical laboratory work the need of a reliable chemical test for replacing the sense perceptions in making the finer discriminations is apparent. To be entirely satisfactory, such a test should be known to be specific either for the compound that supplies the rancid odor or for some accompanying substance that is known to be present in all rancid fats and in rancid fats only. Our present knowledge of the chemistry of rancidity, however, is not sufficient to afford such a test, while the empirical tests currently used are subject to empirical limitations. Thus, the value of the various peroxid tests rests upon the observation that rancid fats in general contain the peroxid radical, yet Kerr and Sorber (*21*) have found that peroxids may be formed before rancidity has developed. Again, all rancid fats contain aldehydes and respond to the Schiff test with decolorized fuchsin, yet Walker (*40*) has found that the results of this test do not always parallel those obtained with the peroxid test. The test which most nearly parallels the organoleptic observations in its results is the Kreis test with phloroglucin-hydrochloric acid; yet even this test, as heretofore made, is given by certain nonrancid cottonseed oils, and, as will appear in the following, by none of the previously identified constituents of rancid fats.

THEORETICAL

It is a matter of common knowledge that samples of oleic acid acquire a rancid odor on exposure to air and light, and it has been found in this laboratory that the rancid acid responds to the chemical tests generally used for the detection of rancidity in fats. This fact, together with the fact that neither stearic acid nor glycerin behaves in a similar manner, would seem to indicate that the oleic acid radical is the point of attack in the development of rancidity, and that a study of the chemistry of rancidity should begin with a study of the oxidation of oleic acid.

A considerable number of references to the oxidation of oleic acid are to be found in the literature. The mechanism of the process and the products obtained seem to depend somewhat upon the oxidizing agent used and the conditions of the experiment. The more important results are mentioned below.

On oxidation of oleic acid with alkaline potassium permanganate Saytzeff (*34*) obtained dihydroxystearic acid as the initial product, together with small amounts of azelaic and volatile fatty acids, formed, presumably, by decomposition of the dihydroxystearic acid. Holde and Marcusson (*18*), following a somewhat more vigorous oxidation with a less alkaline permanganate solution, were able to isolate a small amount

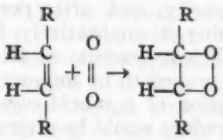
of ketoxystearic acid, while Nicolet and Jurist (*26*) have prepared diketostearic acid by oxidizing oleic acid with chromic anhydrid in acetic acid solution.

Employing a more vigorous oxidation with nitric acid, Redtenbacher (*31*) noted the formation of volatile fatty acids and dicarboxylic acids; Carette (*6*) identified glutaric acid; Laurent (*23, p. 166*) detected heptylic, suberic, pimelenic, adipic, and azelaic acids; Bromeis (*4*) obtained suberic, pimelenic, and adipic acids; while Arppe (*1*) has noted the formation of azelaic acid.

Harries and Thieme (*38*) have studied the action of ozone on oleic acid and have found that the initial reaction consists in the addition of ozone across the double bond with the formation of oleic acid ozonid. The isolated ozonid is described as being a comparatively unstable, sirupy liquid of penetrating odor, which, when subjected to hydrolysis, breaks down into azelaic half aldehyde and pelargonic aldehyde (or their peroxids), which may in turn be oxidized to the corresponding acids.

Many of the above-mentioned oxidation products of oleic acid have been observed both in fats and in oleic acid after the spontaneous development of rancidity. Scala in particular has identified formic, acetic, butyric, heptylic, azelaic, and apparently dihydroxystearic acids as products of the atmospheric oxidation of oleic acid (*36*), and has recovered pelargonic, heptylic, caproic, and butyric aldehydes as well as the corresponding acids and formic acid from a specimen of rancid fat (*37*); while Nicolet and Liddle (*27*) have isolated as much as 10 per cent of azelaic acid from a sample of rancid cottonseed oil. It is surprising that atmospheric oxygen is able so completely to reproduce the profound changes effected artificially in the oleic acid molecule by the more vigorous oxidizing agents mentioned above.

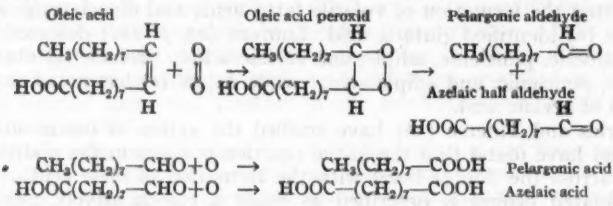
Engler and Weissberg (*11*) have shown, however, that ordinary molecular oxygen may attack the double bond in much the same manner as may ozone. In this process, known as autoxidation, the oxygen is added molecularly across the double bond with the formation of a particular type of peroxid which they term moloxid:



While the isolation of these peroxids presents many difficulties, a few of them have been prepared and the existence of many others seems to have been fairly well established. In properties they are said to resemble the ozonids described by Harries and Thieme (*38*), being characterized by instability and generally, according to Durrans (*9*), by a penetrating odor due to intramolecular stress. Like the ozonids, they readily undergo cleavage with the formation of aldehydes. Engler and Weissberg (*11*) classify the unsaturated fatty acids as compounds that are subject to autoxidation.

It appeared, therefore, that the development of rancidity in fats might be attributed provisionally, as had already been suggested by Winckel

(*41*) and by Heffter (*17*),[3] to an autoxidation of the oleic acid radical, followed by a rupture of the moloxid thus formed and further oxidation of its cleavage products. These changes might be formulated somewhat as follows:



$$CH_3(CH_2)_7—CHO + O \rightarrow CH_3(CH_2)_7—COOH$$ Pelargonic acid

$$HOOC(CH_2)_7—CHO + O \rightarrow HOOC—(CH_2)_7—COOH$$ Azelaic acid

Such a scheme would readily account for the peroxid reaction of rancid fats, as well as for the presence of pelargonic aldehyde, pelargonic acid, azelaic half aldehyde, and azelaic acid. While the other aldehydes and acids, whether monobasic or dibasic, previously detected in rancid fats would be more difficult to account for on the basis of this scheme, one might postulate that they owe their origin to the presence in the original fat of isomers of oleic acid, or to a migration of the double bond during the oxidation. Still other secondary reactions might be pictured whereby the oleic acid moloxid is rearranged to form ketoxystearic acid or reacts with unchanged oleic acid or with another molecule of the moloxid to form dihydroxystearic acid or diketostearic acid.

While these ideas are not fully borne out by the work which follows, they formed the point of attack for the present investigation. At the outset it was confidently expected that the substance responsible for the rancid odor and for the Kreis test would fit in with this scheme, and in this connection azelaic half aldehyde, pelargonic aldehyde, diketostearic acid, and ketoxystearic acid were suspected in particular.

EXPERIMENTAL

It was originally planned to prepare a considerable quantity of oleic acid of unquestionable purity, and, after permitting it to become intensely rancid, to examine it qualitatively for the products formed. The preparation of a sufficient quantity of oleic acid of the desired degree of purity, however, proved to be impracticable; also a preliminary attempt at the fractionation of a rancid oleic acid indicated that the analytical method of procedure would be attended with great difficulties, such as might be expected in the separation of small quantities of homologous acids and aldehydes having but slight differences in physical and chemical properties. The original plan was therefore abandoned, and a systematic search was made among the known degradation products of oleic acid for the substance or substances characteristic of the rancid condition, a search which ultimately suggested a compound not previously recognized as a derivative of oleic acid.

EXAMINATION OF KNOWN OXIDATION PRODUCTS OF OLEIC ACID

The known oxidation products of oleic acid were examined in a preliminary way as to odor and as to behavior with the Kreis phloroglucin-

[3] A recent article by R. H. Kerr and D. G. Sorber (*21*) similarly points to the formation of peroxids of the unsaturated fatty acids as the initial stage in the development of rancidity, although oleic acid is not mentioned specifically.

hydrochloric acid reagent, since these tests are probably the two most dependable and widely used criteria of the rancid condition. No rigorous purification of these compounds was attempted, on the principle that the presence of an impurity, even in appreciable amounts, would be more apt to add to, than to detract from, the odor and reactivity of the substance under examination. In each case the tests were made both on the substance itself and on a perfectly sweet fat to which the substance had been added.

The Kreis test for rancidity was performed as follows:

Five cc. of fresh fat to which the compound to be examined had been added, or a few drops of a dilute ethereal or aqueous solution of the compound itself, were shaken with 5 cc. of concentrated hydrochloric acid. The mixture was then shaken with 5 cc. of a 1 per cent ethereal solution of phloroglucin. The development of a typical red color in the hydrochloric acid was regarded as constituting a positive reaction.

While it was not anticipated that the fatty acids would respond to the Kreis test, the test was carried out as a routine procedure in all cases.

HYDROXYSTEARIC ACID.—Hydroxystearic acid was prepared by the method of Saytzeff (*35*). Considerable difficulty was experienced in purifying the crude product, but by holding its ethereal solution at a temperature below 0° C. for several days a separation of impure crystals was obtained, after which the product was further purified by recrystallization, once from ether, twice from alcohol, and twice again from ether. The material thus obtained was in the form of small, white, somewhat lustrous crystals, melting at 79° C. as compared with the reported melting point of from 83° to 85° C. for pure hydroxystearic acid. The substance proved to be odorless and failed to respond to the Kreis test, whether tested by itself or in the presence of a nonrancid fat.

DIHYDROXYSTEARIC ACID.—Dihydroxystearic acid was prepared by the method of Saytzeff (*34*). The raw product was recrystallized from hot alcohol several times, whereby a white crystalline material was obtained which melted at 129.8° C., as compared with the melting point of 136.5° C. reported for the completely pure substance. Dihydroxystearic acid proved to be odorless, and failed to respond to the Kreis test, as did also a nonrancid lard to which it had been added.

KETOXYSTEARIC ACID.—Holde and Marcusson (*18*) were able to recover a small amount of ketoxystearic acid from the products formed when oleic acid is oxidized with potassium permanganate in presence of a single equivalent of alkali instead of the large excess of alkali employed in the preparation of dihydroxystearic acid. An attempt to prepare this substance in the manner described by Holde and Mareusson was not successful.

While in view of its ketone group, this compound was among those originally thought of as being responsible for the behavior of rancid fats in the Kreis test and as contributing toward the rancid odor, no further attempt was made to prepare ketoxystearic acid, since the negative results obtained meanwhile with the closely related diketostearic acid, in comparison with which the ketoxystearic acid should be less odorous and less reactive, seemed to indicate that the latter product also must be odorless, and negative in its reaction with the Kreis reagent.

DIKETOSTEARIC ACID.—The preparation of diketostearic acid was first attempted by the method of Overbeck (*28*), but as satisfactory results

were not obtained, a method described by Nicolet and Jurist (*26*) was employed. No difficulty was experienced in obtaining a fairly pure product, in the form of pale, greenish-yellow crystals that melted at 84.9° to 85.3° C., as compared with 86° C., reported in the literature for the pure product.

As already observed, diketostearic acid was found to be odorless. Furthermore, neither the acid itself nor a nonrancid lard to which it had been added responded to the Kreis test.

AZELAIC ACID.—Azelaic acid has been recovered from rancid fats (*27*) and has been obtained as a cleavage product of oleic acid ozonid (*38*). While a certain amount of this product was obtained from oleic acid ozonid in the course of these studies, a larger quantity was obtained as a by-product from the preparation of dihydroxystearic acid.

The acid solution from which the dihydroxystearic acid had been precipitated was concentrated to a small volume and allowed to cool. A mass of white crystals separated, as well as a small amount of oily substance, which floated, for the most part, on the surface of the liquid. The oil was removed mechanically so far as possible, after which the crystals were collected on a filter and repeatedly recrystallized from hot water.

A white, well-crystallized product was obtained which melted at 99.1° C. as compared with 106° C. for pure azelaic acid. The low melting point was presumably due to the incomplete separation of the oily substance, presumably pelargonic acid. The product was odorless, and failed to react in the Kreis test, either alone or in presence of nonrancid lard.

PELARGONIC ACID.—Pelargonic acid has been isolated from rancid fats by Scala (*37*) and has been obtained as a cleavage product of oleic acid ozonid and as one of the products in the oxidation of oleic acid with potassium permanganate.

A sample of this acid boiling at 252° to 255° C., as compared with 252° to 254° C. for the pure product, was obtained through the courtesy of Doctor Spencer of the Bureau of Chemistry of this department. It was found to have a mild odor, and in small quantities was without appreciable effect on the odor of a nonrancid lard to which it had been added. Negative Kreis tests were obtained both on the substance itself and on the lard containing it.

CAPRYLIC ACID.—Caprylic acid has been isolated from rancid fats by Scala (*37*), who was of the opinion, however, that it had nothing to do with the odor of rancidity.

A sample of this compound obtained by purchase was found to boil from 237.2° to 238.5° C., as compared with the reported boiling point of from 236° to 237° C. for the pure substance, and was probably a fairly pure product. Its odor was similar to, but stronger than, that of pelargonic acid, and somewhat suggestive of cocoanut. When added to sweet lard in the amount of 1 per cent it imparted to the lard an odor weaker than that of a rancid control and of a different type. While its presence in rancid fats is not questioned, it would appear that it has nothing to do with the rancid odor. Negative Kreis tests were obtained both with caprylic acid itself and with the lard to which it had been added.

HEPTYLIC ACID.—Heptylic acid has been isolated from a rancid fat by Scala (*37*). A purchased sample distilled for the most part between 221.3° and 222.3° C., as compared with 223° to 223.5° C. for the pure acid. In the amount of 1 per cent it imparted to a nonrancid lard the

characteristic odor possessed by many "sour" fats, but no suggestion of a rancid odor. Negative Kreis tests were obtained both with heptylic acid itself and with the lard to which it had been added. Even though present in rancid fats, therefore, it can scarcely be held to contribute toward their rancid odor or to be responsible for their conduct in the Kreis test.

CAPROIC ACID.—Caproic acid has also been isolated from rancid fats by Scala (*37*). A sample obtained by purchase boiled from 200° to 205° C., as compared with 204.2° to 205° C. for the pure substance. It possessed a strong, disagreeable odor, which was in no way similar, however, to the rancid odor. In the amount of 1 per cent it imparted to a sweet lard a sour odor totally unlike that of a rancid control. Both the acid itself and the lard to which it had been added conducted themselves negatively in the Kreis test. Even though present in rancid fats, it is in no way responsible for their rancid odor nor for their response to the Kreis test.

BUTYRIC ACID.—Butyric acid has been isolated from a rancid fat by Scala (*37*). A purchased sample distilled mostly between 162.5° and 164.1° C., as compared with 162.5° C. for the pure acid. An addition of 0.06 per cent imparted to a nonrancid lard a pronounced odor which was distinctly different from that of a rancid control. Toward the Kreis reagent it conducted itself in the same manner as the acids previously mentioned.

FORMIC AND ACETIC ACIDS.—The odors of these acids were too familiar for a special examination to be required. As a matter of routine procedure, however, these acids were subjected to the Kreis test, both by themselves and after addition to a nonrancid lard, with uniformly negative results.

NONYLIC ALDEHYDE.—Nonylic aldehyde has been noted in rancid fats by Scala (*37*). It has also been obtained by cleavage of oleic acid ozonid and is probably formed by cleavage of oleic acid peroxid. It therefore seemed reasonable to suppose that nonylic aldehyde would form an essential part of the picture of the rancid condition and would be found responsible for at least some of the properties distinctive of rancid fats.

An attempt to prepare nonylic aldehyde by cleavage of oleic acid ozonid yielded a product too impure for direct examination, and too small in quantity for satisfactory purification. A small amount of a 10 per cent alcoholic solution obtained in the meanwhile from another laboratory, with satisfactory assurances as to its purity, was therefore employed. Its odor was distinctly suggestive of the odor of rancidity, but of a rancid odor modified by a somewhat pleasing and fragrant quality. When added in small quantity to a sweet lard it imparted to the lard an odor very suggestive of the rancid odor, but lacking in some indefinable quality. Whether tested by itself or in the presence of nonrancid lard, it failed to give a positive reaction in the Kreis test. Where no fat was present, however, larger amounts of the aldehyde gave rise to the production of a yellow color in the aqueous phase or to the formation of a pale yellow precipitate in the Kreis test.

While nonylic aldehyde, therefore, may be suspected of contributing toward the characteristic odor of rancid fats, it would seem that it is not responsible for their behavior in the Kreis test.

HEPTYLIC ALDEHYDE.—Heptylic aldehyde, of all the compounds thus far noted in rancid fats, has been most prominently mentioned as the probable source of the rancid odor. Scala (*37*), in particular, proved

its presence in the rancid fats which he studied and held it to be responble for their rancid odor.

A purchased sample proved to be somewhat impure and was fractionated, the fraction boiling between 152° and 156° C., as compared with 155° C. for the pure product, being reserved for examination. In substance heptylic aldehyde was found to possess a permeating odor, decidedly suggestive of the rancid odor, but accompanied by a fruity fragrance. When 0.25 per cent of heptylic aldehyde was added to a nonrancid lard an odor was obtained which was described by several judges as a typically rancid odor until the sample was compared with a rancid control. When this comparison was made, however, minor differences in odor were noted, the fat containing the heptylic aldehyde lacking a certain sharpness in odor which was present in the control, and possessing a faint fruity fragrance which the control lacked. Neither the aldehyde itself nor the lard to which it had been added responded to the Kreis test for rancidity.

On the basis of these observations and the findings of Scala, it seems reasonable to suppose that all rancid fats contain heptylic aldehyde, and that this compound is largely, but not entirely, responsible for their characteristic rancid odor, but has no bearing on their conduct in the Kreis test.

BUTYRIC ALDEHYDE.—Butyric aldehyde was also noted by Scala (*37*) in certain specimens of rancid fat. A purchased sample distilled for the most part between 73° and 77° C., the limits recorded for the pure product. Its odor was strong and permeating, and in quantities of 0.08 per cent it imparted a pronounced odor to a previously normal sample of lard. Both the substance itself and the lard to which it had been added, however, possessed an odor totally unlike that of a rancid control. If present in the rancid control, its amount must have been extremely small and its odor masked by that of other constituents. In the Kreis test small amounts of butyric aldehyde gave rise to a deep gold color which gradually changed to a very pale pink, while larger quantities gave rise to an orange-colored precipitate. When a nonrancid lard containing butyric aldehyde was similarly tested, a pale pink color was sometimes obtained, though neither in intensity nor in quality did the color resemble that obtained with rancid fats.

ACETALDEHYDE AND FORMALDEHYDE.—These compounds are common laboratory reagents of familiar odor. The odor which they imparted to a nonrancid lard when added in small quantities was scarcely suggestive of the rancid odor; while neither the substances themselves, nor samples of fresh lard to which they had been added, responded to the Kreis test for rancidity.

AZELAIC HALF ALDEHYDE.—The half aldehyde of azelaic acid has been mentioned by Harries and Thieme (*38*) as a cleavage product of oleic acid ozonid. It is described as being very easily oxidized and correspondingly difficult to isolate, and in two successive attempts to prepare it in this laboratory the product was apparently completely oxidized to azelaic acid.

Harries and Türk (*16*), however, succeeded in isolating azelaic half aldehyde, and have described it as a white solid that is ordinarily possessed of a weak odor, but that gives off a strong rose-like odor when warmed. Theoretical considerations would also seem to indicate that the compound could not contribute toward the odor of rancidity, for, according to Durrans (*9*), the carboxyl group tends to suppress odor, and in such a

compound as azelaic half aldehyde one might expect it to counteract the tendency of the aldehyde group to create odor. On theoretical grounds alone, therefore, one might hazard the prediction that azelaic half aldehyde, in the matter of odor, would stand midway between nonylic aldehyde and the odorless azelaic acid and could contribute nothing additional toward the rancid odor. Similarly, on the basis of the color theory, it would appear that azelaic half aldehyde would be in no better position to yield a colored condensation product with phloroglucin than is nonylic aldehyde, which reacted negatively to the Kreis test. It was concluded, therefore, that if azelaic half aldehyde be formed at any stage in the atmospheric oxidation of oleic acid, it is probably oxidized as rapidly as it is formed; and that in any event it could scarcely contribute appreciably toward the odor of rancid fats or account for their positive reaction in the Kreis test.

Contrary to expectations, therefore, all the compounds thus far examined may be excluded as being responsible for the behavior of rancid fats in the Kreis test. All but nonylic and heptylic aldehydes may be excluded as contributing anything essential toward the rancid odor. These latter substances are admittedly present in some rancid fats and probably in all, and admittedly explain in large measure their characteristic odor. To complete the rancid odor and to account for the Kreis test in rancid fats, however, the presence of at least one more substance would seem to be required.

PREPARATION AND EXAMINATION OF OLEIC ACID OZONID

In the first attempt at the preparation of azelaic half aldehyde, the oleic acid ozonid was observed to have a peculiar, penetrating odor, strongly suggestive of that quality in the odor of rancid fats which is not accounted for by the presence of heptylic and nonylic aldehydes. In the second attempt several other interesting observations were made which call for a somewhat detailed description of the experiment.

PREPARATION OF OLEIC ACID OZONID.—The oleic acid ozonid was prepared in an improvised apparatus, essentially by the method of Harries and Thieme (*38*). The oleic acid employed was part of a specimen which had been prepared in this laboratory from beef fat and rendered colorless and odorless by fractional distillation at a pressure of 0.2 mm. Twenty grams of oleic acid were dissolved in 300 cc. of chloroform, and a slow current of ozonized oxygen was passed through the solution for five successive days of seven hours each, after which ozonization was judged to be complete.

EXAMINATION OF OLEIC ACID OZONID.—A few drops of the chloroform solution of the oleic acid ozonid were tested directly with phloroglucin-hydrochloric acid. A pale yellow color that gradually deepened to an orange red was imparted to the hydrochloric acid layer.

About 1 cc. of the chloroform solution was boiled with several cc. of water until the chloroform had disappeared, the ozonid undoubtedly experiencing a certain amount of hydrolysis. On submitting the residue to the Kreis test a pale yellow color was again obtained, but the color deepened more slowly than before and at the end of 30 minutes had acquired scarcely a tinge of pink.

About 1 cc. of the chloroform solution was warmed gently in a watch glass until the chloroform had disappeared, no water being added. The

residue was then warmed gently with concentrated hydrochloric acid for a few minutes and cooled. On adding the Kreis reagent a brownish-red color developed almost immediately, the color deepening somewhat and becoming more frankly red on standing.

The main portion of the chloroform solution was transferred to a distilling flask and subjected to vacuum distillation for the removal of the chloroform. The residue was a clear, thick, glassy sirup, similar in appearance to the oleic acid ozonid described by Harries and Thieme (*38*). It gave off a penetrating odor, strongly suggestive of rancid fat, and liberated iodin from potassium iodid in the presence of dilute acetic acid.

From the above experiment it would appear that neither the ozonid itself nor its usual hydrolytic products, i. e., the C_9 saturated aldehydes and acids, respond to the Kreis test; but that on contact with concentrated hydrochloric acid it may experience a different kind of decomposition with the formation of a compound that yields a red condensation product with phloroglucin. A possible connection between the presence of active oxygen and the odor of rancid fats is also suggested.

EXAMINATION OF THE CLEAVAGE PRODUCTS OF OLEIC ACID OZONID.—The isolated oleic acid ozonid was hydrolyzed by heating on the steam bath for 30 minutes with 75 cc. of water, the heating being continued until the ozonid, which is heavier than water, had been completely transformed into products lighter than water. The mixture was shaken with ether and the aqueous phase drawn off, after which the ethereal solution was shaken with an aqueous solution of sodium bicarbonate, which, according to Harries and Thieme (*38*), should remove most of the azelaic acid and azelaic half-aldehyde. The study of the sodium bicarbonate solution yielded no interesting results and will not be reported. The material remaining in the ether was recovered by drying the ether solution over anhydrous magnesium sulphate and distilling off the ether at a somewhat reduced pressure. The residue was then subjected to fractional distillation at a pressure of about 0.5 mm., fractions being collected between 80° and 110° C. and between 120° and 150° C. An undistilled residue remained.

The water separated from the cleavage products immediately after hydrolysis was examined for peroxid, the presence of which was indicated by an immediate separation of iodin when a few drops of the liquid were added to a neutral solution of potassium iodid. The Kreis test was not applied until the following day, when a deep gold color with a suggestion of pink was obtained with phloroglucin-hydrochloric acid.

The fraction collected between 80° and 110° C. in the vacuum distillation, which should have contained the nonylic aldheyde, amounted to not over 1 cc. It gave a fairly strong peroxid reaction with potassium iodid in the presence of acetic acid, the acetic acid having been added for solvent purposes. It also gave a good test for aldehydes with decolorized fuchsin and a good Kreis test.

The fraction collected between 120° and 150° C. amounted to about 2 or 3 cc. It was probably composed principally of nonylic acid. It gave a positive test for aldehydes with Schiff's reagent, a weak peroxid test, and a weak Kreis test.

From the above-mentioned results it would appear that in the hydrolysis of oleic acid ozonid small amounts of products other than C_9 aldehydes and acids may be formed, and in particular a compound capable of form-

ing a red condensation product with phloroglucin. The possibility of some relationship between the presence of active oxygen and a positive Kreis test is also suggested.

RELATIONSHIP BETWEEN THE KREIS TEST FOR RANCIDITY AND PEROXID OXYGEN

The literature contains many references to the presence of peroxid oxygen in rancid fats, and Vintilesco and Popesco (*39*) in their test for rancidity use the presence of active oxygen as a criterion of the rancid condition. Kerr and Sorber (*21*) in a recent study have shown a wide parallelism between the results of the Kreis test and those obtained by the test employed by these authors. To avoid risk of error in the premises, however, a number of samples of rancid fats and of rancid oleic acid, all giving positive Kreis tests, were examined for the presence of peroxid oxygen in the following manner:

To a small amount of a 10 per cent solution of potassium iodid (free from iodates and from free iodin) an equal quantity of glacial acetic acid (used for solvent purposes) and a like quantity of the fat to be examined were added. The mixture was shaken vigorously for about a minute and diluted with 5 to 10 volumes of water. The production of a blue color on the addition of starch paste was taken as an indication of the presence of peroxid oxygen in the original fat. A blank test on the reagents was performed simultaneously.

Without exception all samples of rancid fats and of rancid oleic acid that were examined gave a positive peroxid test accompanied by a positive Kreis test, while fresh fats gave neither test.

These observations suggested all the more strongly the possibility that the active oxygen of rancid fats might participate in the production of the red color in the Kreis test, or that the Kreis test might even be a general reaction for peroxids of unsaturated compounds. Such a compound is known to be formed when turpentine is exposed to the air; and a positive response to the Kreis test was in fact obtained from an old sample of turpentine that also responded strongly to the test for peroxid oxygen. Subsequently, however, the sample was found to give weak but positive tests for aldehydes with Schiff's reagent and with ammoniacal silver oxid, so that no legitimate conclusion could be drawn regarding the possible participation of the peroxid group in the reaction with phloroglucin-hydrochloric acid.

Several attempts were therefore made to discover what effect the destruction of the peroxid group in rancid fats might have on their behavior in the Kreis test.

A sample of rancid oleic acid, prepared by aeration of the commercial product, was found to react intensely in the peroxid and in the Kreis tests. About 25 cc. of the sample was then shaken with potassium iodid and acetic acid as in the peroxid test, and the treatment continued for two days, the liberated iodin being titrated from time to time with sodium thiosulphate solution. After addition of water and ether, the ethereal solution of the oleic acid was separated, washed with water, and tested for peroxids and for its behavior in the Kreis test. A weak test for peroxids was still obtained, however, together with a somewhat weakened Kreis test.

In a second experiment an attempt was made to destroy the oleic acid peroxid by hydrolysis. Twenty grams of the rancid sample were boiled

with about 200 cc. of water over night under a reflux condenser. The separated oleic acid caused no immediate liberation of iodin in the peroxid test, though a gradual liberation was soon observed and at the end of several hours the test had become strongly positive. The Kreis test was strongly positive from the first, as was also the aldehyde test with Schiff's reagent.

While this experiment seemed to be unfavorable to the idea that active oxygen is a factor in the production of a positive Kreis test in rancid fats, the evidence which it furnished could not be regarded as conclusive, owing to some question as to whether the peroxid test had indicated a temporary absence of peroxids or the presence of a sluggishly reacting peroxid.

In a third experiment, a sample of rancid fat was boiled with water for several hours, the water being replaced from time to time in order to effect the removal of any hydrogen peroxid that might conceivably retard the decomposition of the organic peroxids. Previous experience had shown that but little of the substance that causes the Kreis test is extracted from rancid fats by hot water. At the end of the hydrolysis, the clear fat, separated from the water, gave an almost completely negative test for peroxids and a practically negative Kreis test, even on standing.

Such experiments as those described above, of course, in no case could have proven that peroxid oxygen is a factor in the phloroglucin-hydrochloric acid reaction of rancid fats. But by showing that this reaction persisted after the destruction of the peroxids, they might have eliminated peroxid oxygen from consideration in this connection. This they have failed to do, and the participation of peroxid oxygen in the phloroglucin-hydrochloric acid reaction of rancid fats must for the present be regarded as a possibility.

In the light of this possibility, all the oxidation products of oleic acid that were originally examined as to odor and as to their response in the Kreis test were reexamined in such a manner that the Kreis test was performed after the substance had been treated with hydrogen peroxid. Also mixtures of these substances were similarly examined, both in presence and in absence of hydrogen peroxid. Uniformly negative results were obtained all in cases.

COLORED CONDENSATION PRODUCTS OF PHLOROGLUCIN

Kobert (*22*), in a study of the phloroglucin-hydrochloric acid reaction, concluded that only compounds containing an allyl group ($-CH_2-CH:CH_2$) or a substituted allyl group ($-CH_2-CH:CRR'$) are capable of forming red condensation products with phloroglucin in the Kreis test, and in support of this contention he mentions a whole series of compounds so constituted that react in this way. Yet the fact that the same reaction is given by vanillin and other compounds which contain no such grouping, seems to destroy the validity of his generalization.

According to von Euler (*12*), who has exhaustively reviewed the literature of this subject, the condensation between aldehydes and phloroglucin generally occurs in several stages. As a rule, the primary condensation products formed from one molecule of aldehyde and two molecules of phloroglucin are said to be colorless, the formation of a colored compound being dependent upon subsequent anhydridization followed by oxidation. In this manner a complicated triple ring system results.

In the Kreis test as employed for the detection of rancid fats, however, the aldehydes of the paraffin series have uniformly failed to give rise to distinctly colored products, even in the presence of hydrogen peroxid, and it would appear that under these conditions anhydridization does not occur with appreciable velocity. Thus it appears logical to suppose that the immediate and intense response of rancid fats in the Kreis test is called forth by the presence of a carbonyl compound which contains its own chromophore group and whose primary condensation product with phloroglucin is already colored, in exception to the general rule.

Nitrogenous chromophores being excluded, it appeared, especially in view of Kobert's work, that the double bond ($>C:C<$) would be the most likely chromophore group. And even though the formation of unsaturated compounds in the cleavage of oleic acid peroxid might be somewhat difficult to explain, for it would seem to involve the introduction of new double bonds into the oleic acid carbon chain prior to any rupture at the peroxid group, the study of the unsaturated aldehydes and the unsaturated ketones in connection with the Kreis test seemed to be indicated. Of these substances the unsaturated aldehydes were examined first.

EXAMINATION OF THE UNSATURATED ALDEHYDES

ACROLEIN.—Of the unsaturated aldehydes, acrolein was studied first, as being the lowest member of the series and one of the easiest to prepare. It was prepared by the method of Bergh (*3*), by distillation from a metal retort of 95 parts of dry glycerin in presence of 5 parts of 85 per cent phosphoric acid, followed by redistillation, drying, and fractionation of the raw product. Twenty gm. of material boiling between 52.5° and 54° C., as compared with 52.4° C. for the pure product, were obtained in this way. It possessed the intensely sharp and penetrating odor and the tear-producing property which are characteristic of acrolein.

When a few drops of a dilute aqueous solution of acrolein were tested in the usual way with phloroglucin-hydrochloric acid a flesh-colored precipitate was obtained. In other tests with smaller amounts of acrolein the hydrochloric acid layer remained colorless or showed at most an extremely faint pink tinge. None of these tests resembled those obtained with rancid fats.

Before discarding the above-mentioned tests a few drops of hydrogen-peroxid solution were added to one of the test tubes. No immediate change occurred, but at the expiration of half an hour it was noticed that the hydrochloric acid layer in the tube receiving the hydrogen peroxid had acquired a very decided, though not an intense, pink color, similar to that obtained with slightly rancid fats in the Kreis test.

The hydrogen peroxid was apparently effecting an oxidation of the condensation product; and in order that an indication might be obtained as to whether the oxidation was occurring in the acrolein or in the phloroglucin radical the following tests were performed:

(*a*) Five cc. of the Kreis reagent were first treated with a few drops of a 3 per cent solution of pure hydrogen peroxid, the mixture being then added to another mixture containing a few drops of dilute acrolein solution and 5 cc. of concentrated hydrochloric acid. The result was the same as when the hydrogen peroxid was added to the finished test, i. e., no immediate reaction, but a slow development of a pink color.

(*b*) A few drops of dilute acrolein solution were treated with an equal quantity of a 3 per cent solution of pure hydrogen peroxid. The mixture was allowed to stand for about one minute, after which 5 cc. of concentrated hydrochloric acid and 5 cc. of the Kreis reagent were added. An intense red color was immediately imparted to the hydrochloric acid layer. In similar tests with larger quantities of acrolein and hydrogen peroxid the condensation product separated as an intensely purple precipitate.

It therefore appears that the hydrogen peroxid exerts its effect most rapidly and most completely when added directly to the acrolein, and that a derivative of acrolein is thereby produced which forms a red-colored condensation product with phloroglucin in presence of hydrochloric acid.

CROTONIC ALDEHYDE.—Crotonic aldehyde was prepared by the method of Delépine as given in Beilstein's "Handbuch der Organischen Chemie" (*2, v. 1, p. 729*). The product was not specially purified, but possessed the described odor and appearance of crotonic aldehyde.

When a dilute aqueous solution of crotonic aldehyde was tested in the usual manner with phloroglucin-hydrochloric acid a deep red color was immediately imparted to the hydrochloric acid layer. When the solution was shaken, however, the color faded rapidly and disappeared within a few minutes. When hydrogen peroxid was added to the fully developed test the red color disappeared as rapidly as it had been formed; and when it was added to the crotonic aldehyde before addition of hydrochloric acid and pholoroglucin a negative test was obtained from the beginning.

The red color obtained in the Kreis test on rancid fats does not fade so readily, and it is evident that crotonic aldehyde is not responsible for the conduct of rancid fats in this test.

From the above experiments, it is evident that all unsaturated aldehydes do not behave similarly in the Kreis test. It already is suggested that increased molecular weight of the aldehyde carries with it an increased tendency toward instability of such color as it may give rise to in the Kreis test, possibly through increased tendency toward rupture of the carbon chain at the double bond. At all events, it was decided to postpone the examination of the higher members of this series until the reaction between the acrolein, hydrogen peroxid, and phloroglucin-hydrochloric acid could be more thoroughly investigated in its relation to the reactions of rancid fats.

SPECTROSCOPIC COMPARISON OF COLORED CONDENSATION PRODUCTS OF PHLOROGLUCIN

Because the product formed by the action of hydrogen peroxid on acrolein gives a more or less permanent red color in the Kreis test, it does not necessarily follow that it is identical with the similarly reacting substance in rancid fats. Kobert has mentioned a whole series of compounds which give a similar reaction; and of the compounds available in this laboratory, vanillin, eugenol, cinnamic aldehyde, and aged turpentine were found to behave in the same manner.

Analytical processes for comparing the substance formed by interaction of acrolein and hydrogen peroxid with the similarly reacting substance in rancid fats, or for comparing the colored condensation products

that they form with phloroglucin, were out of the question, because of the impracticability of isolating the reactive substance from rancid fats, or of preparing its condensation product in a sufficiently pure condition. Spectroscopic comparison of the colored condensation products was therefore resorted to. The validity of the method was established by extending the spectroscopic comparisons to the corresponding condensation products of vanillin, eugenol, cinnamic aldehyde and aged turpentine.

Twenty-five cubic centimeters of a rancid sample of commercial oleic acid were shaken in a separatory funnel with 25 cc. of concentrated hydrochloric acid, after which the mixture was shaken with 25 cc. of a 1 per cent ethereal solution of phloroglucin. A portion of the red-colored hydrochloric acid layer was drawn off into a glass cell for spectroscopic comparison.

Corresponding solutions were prepared from samples of rancid lard, rancid cottonseed oil, rancid oleo oil, vanillin, eugenol, aged turpentine, and cinnamic aldehyde, as well as from an acrolein solution to which hydrogen peroxid had been added. Care was used in each case to employ such quantity of the given material as to produce in the hydrochloric acid layer a color of about the same intensity as that of the solution obtained from rancid oleic acid. Minor differences in intensity were adjusted by suitable dilution of the colored solutions with concentrated hydrochloric acid, or by varying the thickness of the layer of solution examined.

The red solutions thus obtained were viewed by transmitted light, two at a time, through a spectroscope designed for such comparisons. The spectra obtained from the solutions prepared from the several rancid fats, from rancid oleic acid, and from the acrolein-hydrogen peroxid mixture appeared to be identical. In each of these cases the spectrum exhibited a fairly narrow, well-defined, apparently symmetrical absorption band, centered at 6.0 on the arbitrary scale of the instrument, and located in the yellow-green region to the right of the D line. The position of the band was identical in each case, at least to within the limits of error of the instrument.

The spectra of the solutions prepared from vanillin, turpentine, eugenol, and cinnamic aldehyde, while showing slight differences among themselves, were similar in type and showed a general absorption beginning at a point in the yellow-green or the blue-green region and extending to the violet end of the spectrum. In no case was a localized band observed similar to that which had been observed in the spectra of the solutions prepared from rancid fats and from acrolein-hydrogen peroxid mixtures. The coloring matters were extracted with amyl alcohol and the solutions thus obtained were reexamined, but no change in the type of the spectrum could be noted in any case, and no significant changes in the length of the darkened areas.

A sample of nonrancid cottonseed oil which gave a red color in the Kreis test was similarly treated and examined. The spectrum obtained resembled those previously obtained in the work with the aromatic substances, and showed no similarity whatever to those obtained in the experiments with rancid fats, rancid oleic acid, or mixtures of acrolein and hydrogen peroxid.

From the above-described experiment it follows that the spectroscopic examination affords a valid means of distinguishing the colored substance formed in the Kreis test on rancid fats from other red condensation

products of phloroglucin; that, so far as could be determined by means of the spectroscope employed, the constituent of rancid fats responsible for the Kreis test forms the same condensation product with phloroglucin as does the compound that results from the interaction of acrolein and hydrogen peroxid; and that the Kreis test, when followed by a spectroscopic examination, is a valid index of rancidity in cottonseed oils, although of doubtful significance in this connection when the spectroscopic examination is omitted.

It remains to determine the chemical constitution and physical properties of this compound formed by interaction of acrolein and hydrogen peroxid, and whether or not it occurs as such in rancid fats or is first liberated under the influence of the concentrated hydrochloric acid used in making the Kreis test. In the interest of brevity, this substance, formed by interaction of acrolein and hydrogen peroxid and giving a red color in the Kreis test, will be referred to hereafter as Substance K.

REACTION BETWEEN ACROLEIN AND HYDROGEN PEROXID

The reaction between acrolein and hydrogen peroxid is apparently not described in the literature. In the study of this reaction it seemed important, first of all, to determine whether it is the double bond or the aldehyde group of the acrolein that is involved in the formation of substance K. Oxidation of the aldehyde group would be indicated by the loss of aldehydic functions, or the acquisition of acidic functions, and could probably be effected by oxidizing agents other than hydrogen peroxid. These questions were examined in the following experiments:

(*a*) A small amount of acrolein solution in a test tube was treated with an excess of N/10 potassium permanganate solution in presence of a small amount of dilute sulphuric acid. The pink color was dispelled by adding the required amount of dilute oxalic acid solution. On testing a few drops of the resulting solution with phloroglucin-hydrochloric acid no red color was obtained.

(*b*) According to Beilstein's "Handbuch der Organischen Chemie" (*2, v. 2, p. 389*), acrolein is oxidized to acrylic acid by the action of freshly prepared silver oxid in the presence of light.

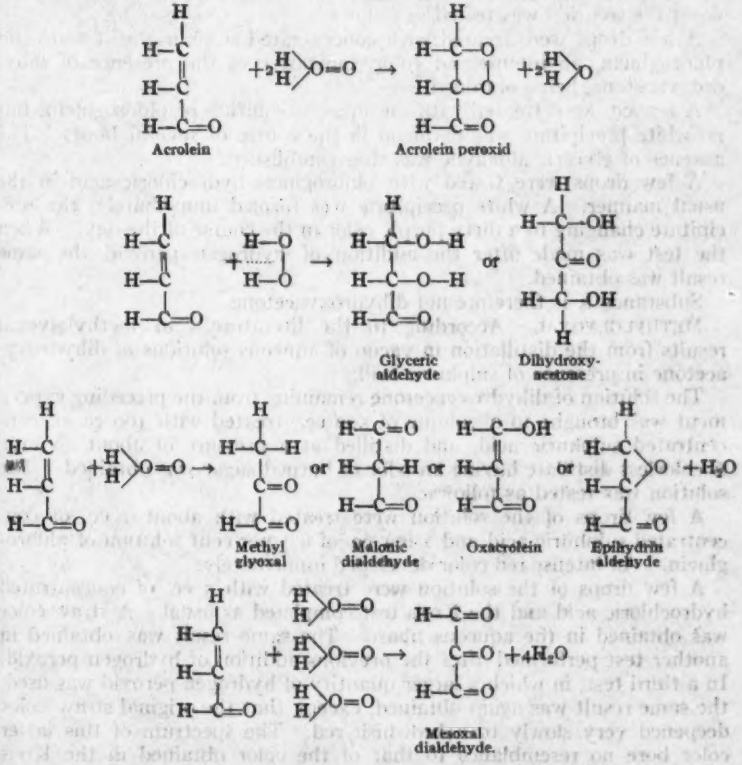
Ten gm. of freshly prepared silver oxid were suspended in a solution of 10 cc. of acrolein in 100 cc. of water, and the mixture was exposed in a west window for several days. Acid formation had occurred, as shown by the effervescence that took place when sodium bicarbonate was added. The acrylic acid was lost by accident at a subsequent stage of the experiment, however, and could not, therefore, be thoroughly identified.

Absence of substance K was indicated by failure of the acid solution to respond to the Kreis test. Some unchanged acrolein remained in solution, as indicated by the odor and by a positive test for aldehydes with decolorized fuchsin.

(*c*) A purchased sample of acrylic acid, bearing the Kahlbaum label, was tested with phloroglucin-hydrochloric acid, both with and without the previous addition of hydrogen peroxid, a negative test being obtained in each case.

From the above-described experiments it would appear that substance K is not acrylic acid, and that the reaction leading to the formation of substance K is not brought about by oxidizing reagents in general, but depends upon the chemical nature of peroxid oxygen in particular.

This would seem to indicate that the hydrogen peroxid reacts with the double bond of acrolein to form a compound that still contains the aldehyde group. Many such reactions might be imagined, the products varying somewhat with the quantities of the respective substances that are involved. Thus, for the formation of acrolein moloxid, two molecules of hydrogen peroxid would be required for one of acrolein; whereas for the formation of glyceric aldehyde, methyl glyoxal, oxyacrolein, malonic dialdehyde, or epihydrin aldehyde, one molecule of hydrogen peroxid would suffice; and for the formation of mesoxaldialdehyde three would be needed. Without considering for the present a large number of more complicated reaction products that might conceivably be formed, these substances were examined as to their behavior in the Kreis test, so far as practicable or desirable.



GLYCERIC ALDEHYDE.—The condensation reaction between glyceric aldehyde and phloroglucin has already been studied by Wohl and Neuberg (*45*). According to these authors, the only product that is formed, with any degree of readiness at least, is a white, crystalline substance, insoluble in water and fairly easily formed in aqueous solutions of the reagents even when condensing agents are not employed. While the possible presence of glyceric aldehyde in rancid coconut oil has been

suggested by Walker (*40*), it is evidently not responsible for the reaction of rancid fats in the Kreis test, nor identical with substance K.

DIHYDROXYACETONE.—In most of the reactions leading to the formation of glyceric aldehyde the isomeric dihydroxyacetone is also formed. It is said to yield brilliant colors with phloroglucin in presence of concentrated sulphuric acid. It was prepared by the method of Fischer and Tafel (*15*), which was originally supposed to lead to the formation of glyceric aldehyde, but which has since been shown by Wohl and Neuberg (*45*) to lead to the formation of dihydroxyacetone only.

Ten parts of glycerin were mixed with 60 parts of water and 35 parts of crystalline sodium bicarbonate. The mixture was brought to a temperature of 10° C., after which 15 parts of bromin were added from a burette. At the end of 30 minutes the mixture was acidified and the liberated bromin reduced with sulphur dioxid gas. After standing overnight the solution was tested as follows:

A few drops were treated with concentrated sulphuric acid and solid phloroglucin, an intense red color, indicative of the presence of dihydroxyacetone, being obtained.

A few cc. were treated with an aqueous solution of phloroglucin, but no white precipitate was obtained in the course of several hours. The absence of glyceric aldehyde was thus established.

A few drops were tested with phloroglucin-hydrochloric acid in the usual manner. A white precipitate was formed immediately, the precipitate changing to a dirty purple color in the course of the day. When the test was made after the addition of hydrogen peroxid the same result was obtained.

Substance K is therefore not dihydroxyacetone.

METHYLGLYOXAL.—According to the literature (*30*) methylglyoxal results from the distillation in vacuo of aqueous solutions of dihydroxyacetone in presence of sulphuric acid.

The solution of dihydroxyacetone remaining from the preceding experiment was brought to a volume of 500 cc., treated with 100 cc. of concentrated sulphuric acid, and distilled at a pressure of about 20 mm. A colorless distillate having an odor of burned sugar was obtained. The solution was tested as follows:

A few drops of the solution were treated with about 1 cc. of concentrated sulphuric acid and a few cc. of a 1 per cent solution of phloroglucin. An intense red color developed immediately.

A few drops of the solution were treated with 5 cc. of concentrated hydrochloric acid and the Kreis test completed as usual. A straw color was obtained in the aqueous phase. The same result was obtained in another test performed after the previous addition of hydrogen peroxid. In a third test, in which a larger quantity of hydrogen peroxid was used, the same result was again obtained, except that the original straw color deepened very slowly to a brownish red. The spectrum of this latter color bore no resemblance to that of the color obtained in the Kreis test on rancid fats and on mixtures of acrolein and hydrogen peroxid.

Whether or not methylglyoxal be formed in the reaction between acrolein and hydrogen peroxid, it is not the same as substance K in which we are now interested.

OXYACROLEIN AND MALONICDIALDEHYDE.—According to the literature (*7*) these substances are known only in aqueous solution and are tautomeric. An intensely red color is said to be obtained when their aqueous solution is treated with ferric chlorid. No such reaction could

be obtained, however, with mixtures of acrolein and hydrogen peroxid. Whatever the conduct of the substances in question might be in the Kreis test, they are apparently not formed by interaction of acrolein and hydrogen peroxid and are of no interest in the present connection.

ACROLEIN PEROXID.—Of the substances mentioned above, all but acrolein peroxid, mesoxaldialdehyde and epihydrin aldehyde have been eliminated as factors in the formation of the red color obtained in the Kreis test on mixtures of acrolein and hydrogen peroxid. Of these substances, acrolein peroxid was considered most seriously, partly because Moureu and Dufraisse (*25*) have pointed out the readiness with which acrolein forms a peroxid, partly because of a suspicion that peroxid oxygen might participate in the reaction between rancid fats and the Kreis reagent, and partly because Pastureau and Launay (*29*) had previously prepared the peroxid of mesityl oxid by use of hydrogen peroxid in acid solution. Two molecules of hydrogen peroxid to one of acrolein would be required for the formation of acrolein peroxid, whereas three would be required for the formation of mesoxaldialdehyde and but one for the formation of epihydrin aldehyde. The following experiment was undertaken in an attempt to obtain a rough indication of the amount of hydrogen peroxid that actually enters into the reaction.

Six mixtures of acrolein and hydrogen peroxid in varying proportions were diluted with water to 25 cc., and six similar mixtures were brought to a like volume by addition of concentrated hydrochloric acid. Table I indicates the weights of freshly prepared and rectified acrolein and of pure 30 per cent hydrogen peroxid entering into the respective solutions, together with the approximate number of molecules of hydrogen peroxid per molecule of acrolein.

TABLE I.—*Composition of solutions of acrolein and hydrogen peroxid*

No.	A. Aqueous solutions.			No.	B. Concentrated hydrochloric acid solutions.		
	Weight of acrolein.	Weight of 30 per cent solution of hydrogen peroxid.	Mol. H_2O_2 per mol. acrolein.		Weight of acrolein.	Weight of 30 per cent solution of hydrogen peroxide.	Mol. H_2O_2 per mol. acrolein.
	Gm.	*Gm.*			*Gm.*	*Gm.*	
1	0.58	0.6025	0.5+	1*a*	0.5344	0.5461	0.5+
2	.5319	1.3420	1.2+	2*a*	.6094	1.2993	1.0+
3	.4558	1.9692	2.1+	3*a*	.5593	2.2384	2.0−
4	.4909	3.0495	3.0+	4*a*	.5126	3.0688	2.9+
5	.5175	4.0281	3.8+	5*a*	.5404	4.3598	4.0−
6	.6291	6.3664	5.0−	6*a*	.5583	5.5620	4.9+

All of the aqueous solutions remained water clear on dilution. Nos. 1 and 2 were faintly alkaline to methyl red, while Nos. 5 and 6 were faintly acid. In the second series a cloudiness, which soon developed to a bulky, cream-colored precipitate, was formed in solutions 1*a*, 2*a*, and 3*a*, on addition of the hydrochloric acid, while the other solutions remained clear; but the color of all the solutions of the acid series gradually darkened to yellowish brown.

Within 15 minutes after their preparation each of the 12 solutions was subjected to the Kreis test and to the peroxid test with potassium iodid.

The peroxid tests were all made without addition of acid, under which conditions the hydrogen peroxid employed in this experiment reacted negatively. The results of these tests were as follows:

TABLE II.—*Results of tests of solutions of acrolein and hydrogen peroxid*

No.	Kreis test.	Peroxid test.
1	Negative	Negative.
2	do	Do.
3	Strongly positive	Strongly positive.
4	do	Do.
5	do	Do.
6	do	Do.
1a to 6a	All negative	All negative.

The hydrochloric acid series was not tested further; but at the expiration of 22 hours the water series was tested again, with the same results as before. Addition of hydrogen peroxid to solution 2, until the solution contained a total of three and one-half molecules of hydrogen peroxid per molecule of acrolein, failed to elicit a positive response to the Kreis test.

The results of this experiment were quite surprising. It was to have been expected that substance K would still be formed, though in diminished quantity, in solutions containing less than a stoichiometric quantity of hydrogen peroxid. But as a repetition of these tests (with the same material, to be sure) led to similar results, it was concluded (erroneously, as will appear later), that substance K is formed in an irreversible reaction from two molecules of hydrogen peroxid and one of acrolein, that it contains peroxid oxygen, and that it must therefore be acrolein peroxid. It appeared, furthermore, that the concentrated hydrochloric acid favored the destruction of substance K, and probably functioned, not in the formation of substance K, but merely in the condensation reaction with phloroglucin.

On the basis of this experiment many unsuccessful attempts were made to isolate substance K from aqueous solutions of acrolein and hydrogen peroxid. Evaporation of such solutions in a vacuum desiccator led to small, colorless, sirupy residues, possessing no characteristic odor, and reacting but weakly with the Kreis reagent. Distillation in vacuo led to similar residues, though positive Kreis tests could be obtained on the aqueous distillates. The tests on the distillates, however, were always weaker than those obtained on the original solutions, and could also be variously interpreted as indicating the volatility of substance K as such, or as pointing to a gradual decomposition of substance K into acrolein and hydrogen peroxid and the separate distillation of these compounds. Finally, an attempt was made to prepare acrolein peroxid by the method employed by Pastureau and Launay (*29*) in the preparation of the peroxid of mesityl oxid, that is, by subjecting acrolein, instead of mesityl oxid, to the action of 3 per cent hydrogen peroxid in dilute sulphuric acid solution. An abundant, white, flocculent precipitate was obtained, which failed to dissolve in the usual solvents, and was apparently a polymerization product of acrolein.

In the course of this work, a number of observations were made that could not be reconciled with the previous finding that substance K is not

formed in solutions containing less than two molecules of hydrogen peroxid per molecule of acrolein. The experiment in which this result was obtained was therefore repeated, a new preparation of acrolein being employed; but the original results could not be duplicated. The smallest addition of hydrogen peroxid to an acrolein solution resulted now in a positive Kreis test; and the peculiar results obtained in the original experiment were traced to the slight and accidental alkalinity of solutions 1 and 2. The conclusions reached on the basis of the original experiment were therefore unjustified.

Attention was again turned to the combining proportion of acrolein and hydrogen peroxid in the reaction:

$$\text{Acrolein} + \text{hydrogen peroxid} \rightarrow \text{substance K.}$$

Should this reaction proceed to completion, this proportion could probably be determined by preparing solutions containing like concentrations of acrolein and various concentrations of hydrogen peroxid and noting the smallest concentration of hydrogen peroxid required for the maximum production of color in the Kreis test. If, however, the reaction stops short of completion, uncombined acrolein and hydrogen peroxid would always remain in solution, and so long as this condition obtained, the colorimetric method could yield no information as to their combining proportion.

With the idea of determining whether or not the reaction proceeds to completion, a solution containing 0.41 molecules of hydrogen peroxid per molecule of acrolein was examined as follows for free hydrogen peroxid:

(*a*) A 10 cc. aliquot of this solution, representing an original addition of hydrogen peroxid equivalent to 22 cc. of tenth-normal solution, was titrated with tenth-normal potassium permanganate in an attempt to determine, if possible, the unused hydrogen peroxid. A sharp end point could not be obtained. The solution was still uncolored, and reacted positively in the Kreis test after 22 cc. of permanganate had been added, but gave a negative Kreis test after 35 cc. had been added. Apparently the permanganate was reacting with the organic material as well as with free hydrogen peroxid.

(*b*) When potassium iodid was added to 0.5 cc. of the same acrolein-hydrogen peroxid solution in neutral reaction, the presence of an organic peroxid was indicated by a liberation of iodin. After the solution had been decolorized with thiosulphate, the addition of acid called forth a fresh liberation of iodin, and the presence of free hydrogen peroxid was indicated. The total quantity of iodin liberated in the two stages was equivalent to 3.35 cc. of a nearly tenth-normal thiosulphate solution, or more than equivalent to the originally added hydrogen peroxid. The presence of uncombined hydrogen peroxid, which was confirmed by a chromic acid test, indicates that the reaction

$$\text{Acrolein} + \text{hydrogen peroxid} \rightarrow \text{substance K}$$

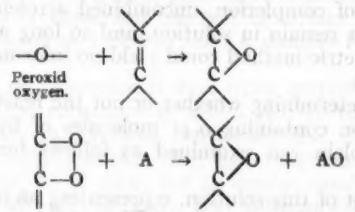
does not proceed to completion, in neutral or in weakly acid solution at least.

(*c*) A 0.5 cc. aliquot of the same acrolein-hydrogen peroxid solution was added to 5 cc. of concentrated hydrochloric acid, after which the acid solution was treated with potassium iodid. The iodin liberated was equivalent to but 1.95 cc. of the same thiosulphate solution, as compared

with the 3.35 cc. required in the preceding test. The titrated solution gave a strongly positive Kreis test.

Repetition of Experiments *b* and *c* showed beyond doubt that when any neutral acrolein-hydrogen peroxid solution is treated successively with potassium iodid, excess hydrochloric acid and thiosulphate, in the order named, the resultant solution does not respond to the Kreis test. But when the order is changed so that a large excess of concentrated hydrochloric acid is added first and the other reagents afterwards, a positive test is obtained. Evidently substance K is not destroyed by the removal of peroxid oxygen and is not formed except in presence of a considerable concentration of acid. It may be definitely stated, therefore, that substance K does not contain a peroxid group and is not acrolein peroxid.

EPIHYDRIN ALDEHYDE.—*A priori*, it was perhaps to have been expected that epihydrin aldehyde should be formed in the reaction between hydrogen peroxid and acrolein, more especially since other ethylene oxids are known to be formed in analogous reactions between peroxid oxygen and the ethylene linkage. Such a reaction, too, might occur in rancid fats, where also an ethylene oxid might result by degradation of a peroxid.



But in spite of these relationships the absence of any description of epihydrin aldehyde in the literature consulted, led to the belief that this compound must be extremely unstable and that, even if formed in the reaction between acrolein and hydrogen peroxid, it immediately would be converted, through addition of water, into glyceric aldehyde.

It now appears, however, that of the C_3 compounds originally suggested as being possibly identical with substance K, all but epihydrin aldehyde and mesoxaldialdehyde have been eliminated; and of these compounds, epihydrin aldehyde, being in effect a simple acrolein oxid, would seem to be the more closely related to acrolein.

While epihydrin aldehyde itself seems never to have been prepared, two of its acetals have been synthesized, the diethylacetal by Wohl (*43*), and the dimethylacetal by Wohl and Momber (*44*). The latter authors also attempted to prepare the dimethylacetal by the direct action of various peroxids on the corresponding acrolein acetal, but were unsuccessful in their efforts.

The preparation of epihydrin aldehyde diethylacetal by the process employed by Wohl was eventually undertaken, the reactions involved being as follows:

I $CH_2{=}CH{-}CHO + HCl + 2C_2H_5OH \rightarrow CH_2Cl{-}CH_2{-}CH(OC_2H_5)_2 + H_2O$
(Acrolein) (β-Chlor-propionic aldehyde diethylacetal)

II $CH_2Cl{-}CH_2{-}CH(OC_2H_5)_2 + KOH \rightarrow CH_2{:}CH{-}CH(OC_2H_5)_2 + KCl + H_2O$
(Acrolein diethylacetal)

III $CH_2{:}CH{-}CH(OC_2H_5)_2 + HOCl \rightarrow CH_2OH{-}CHCl{-}CH(OC_2H_5)_2$
(α-Chlor-β-oxy-propionic aldehyde diethylacetal)

IV $CH_2OH{-}CHCl{-}CH(OC_2H_5)_2 + KOH \rightarrow CH_2{-}\underset{\displaystyle O}{\underset{\vert}{CH}}{-}CH(OC_2H_5)_2 + KCl + H_2O$
(Epihydrin aldehyde diethylacetal)

The original attempts to carry out this synthesis were almost entirely unsuccessful, owing, in part at least, to the scantiness of Wohl's description of his precise working conditions. Eventually, however, a successful preparation was accomplished by adopting Witzemann's (*42*) modifications for the first and second stages and those of Wohl and Schweitzer (*46*) for the third stage, while the procedure adopted for the fourth stage was essentially an adaptation of that employed by Wohl and Momber (*44*) in their preparation of the dimethylacetal of epihydrin aldehyde. In connection with these citations it should be further observed, perhaps, that mechanical agitation was employed during the reactions of the first and third stages, that in all processes calling for sharp cooling the temperature was maintained between $-2°$ and $+2°$ C., and that each of the intermediate products was dried and purified by fractional distillation before being employed in the subsequent stage.

Particular care, also, was exercised in the preparation of the pulverized caustic potash employed in reactions II and IV, as the success of the synthesis was found to depend largely upon this point. The commercial potassium hydroxid "purified by alcohol," was first dried by fusion in a silver crucible, after which it was pulverized and sieved in a dehumidified chamber in current use in these laboratories. The sieved material was then stored for several days under absolute ether in presence of metallic sodium, after which the ether was removed in vacuo and the metallic sodium separated by means of a sieve. In the first distillation with caustic potash, the use of a distilling column was omitted as occasioning the application of an unnecessarily high heat to the contents of the distilling flask, and this omission seemed to improve the yield and quality of the acrolein diethylacetal obtained in the distillate.

In carrying out the final reaction, the β-oxy-α-chlor-propionic aldehyde diethylacetal was first diluted with an equal weight of absolute ether and then treated with an equal weight of pulverized caustic potash. The mixture thus obtained was mechanically better adapted for distillation than that obtained when no ether and double the amount of caustic potash is employed. In the ensuing distillation the apparatus was gradually evacuated to a pressure of 0.2 mm., the ether being condensed in a trap cooled with liquid air, while the higher boiling fractions were condensed in a condenser cooled with cold brine and collected in a receiver packed in a freezing mixture. But one fraction, boiling between 44° and 60° C., was collected.

By fractionation of the final crude product, about 20 cc. of apparently pure epihydrin aldehyde diethylacetal were obtained. Like Wohl's product, it was practically insoluble in water and readily decomposed by acids, while it boiled undecomposed at atmospheric pressure between 165° and 168° C. as compared with 165° C. for Wohl's preparation. In neutral solution it gave a negative reaction for aldehydes with Schiff's reagent; but after a short treatment with very dilute acid, followed by neutralization of the acid, a positive test for aldehydes was obtained. The acetal possessed a rather agreeable odor that is difficult to describe.

When a few drops of a dilute ethereal solution of epihydrin aldehyde diethylacetal were tested in the usual manner with concentrated hydrochloric acid and phloroglucin, a strong red color was immediately imparted to the hydrochloric acid solution. A similar though slower response was obtained when the test was performed with dilute (about 1:20) hydrochloric acid instead of the concentrated acid; but when no

acid was employed, no color was obtained. Evidently, the acid serves first of all to hydrolyze the acetal and liberate free epihydrin aldehyde, which then condenses with phloroglucin even in weak acid solution, in the same manner as does the product of the reaction between acrolein and hydrogen peroxid.

The colored phloroglucids obtained from epihydrin aldehyde diethylacetal on one hand, and from mixtures of acrolein and hydrogen peroxid on the other, were compared spectroscopically in concentrated hydrochloric acid solution. The spectra of the two solutions appeared to be identical, each spectrum exhibiting a well-defined band centered at 6.0 on the arbitrary scale of the instrument.

While the foregoing experiments point strongly toward the conclusion that substance K is identical with epihydrin aldehyde, the evidence is not yet final. There is still the possibility that the spectroscope employed may not have been delicate enough to show minor differences between two nearly identical spectra. Again, it would be conceivable that epihydrin aldehyde might undergo some transformation in presence of hydrochloric acid, and that the colored phloroglucid is derived from some secondary decomposition product thus formed rather than from the epihydrin aldehyde itself.

As to the first of these possibilities it may be said that the results of the spectroscopic examination were completely confirmed by use of a very delicate spectrophotometer, the results obtained thereby being described in another section of this paper. The identity of the colored phloroglucids obtained from substance K and from epihydrin aldehyde is therefore beyond question.

The second of the possibilities suggested above, viz, that the epihydrin aldehyde, under the conditions of the Kreis test, might experience some indeterminate change prior to its condensation with phloroglucin, seemed to be rather remote, since such carbonyl compounds as might conceivably result from the decomposition or rearrangement of epihydrin aldehyde have already been eliminated as factors in the production of the red phloroglucid which we have described. The desirability of obtaining analytical evidence regarding the composition of this compound is nevertheless apparent; and a small amount of the phloroglucid was prepared from acrolein and hydrogen peroxid, and submitted to analysis by combustion.

Twenty-five cc. of a 5 per cent aqueous solution of freshly prepared acrolein were treated with an excess of pure 30 per cent hydrogen peroxid and about 300 cc. of concentrated hydrochloric acid. Three hundred cubic centimeters of a 1 per cent ethereal solution of phloroglucin were added immediately, and the mixture was shaken in a separatory funnel. An intense red color was immediately imparted to the hydrochloric acid layer, followed rapidly by the separation of a deep purple precipitate. The hydrochloric acid layer containing the precipitate was separated from the ethereal phase, diluted with about 500 cc. of water, separated from the ether that was thrown out of solution, and filtered. The precipitate, which had been collected on a Buchner funnel, was washed thoroughly with water, air dried, and tested for solubility with a view to its recrystallization. It proved to be insoluble in alcohol, ether, benzene, chloroform, acetone, carbon tetrachlorid, carbon bisulphid, isoamyl alcohol, butyl alcohol, methyl alcohol, petroleum ether, and pyridin; very slightly soluble in glacial acetic acid and in concentrated

hydrochloric acid; and somewhat more soluble, possibly with decomposition, in alkaline solutions. The solubility in all of these solvents seemed to be too small for purposes of crystallization. The substance also appeared to char before it melted. No indication could be obtained, therefore, as to its probable purity. It was nevertheless dried to constant weight at a temperature of 105° C. and analyzed with the following results:

	C.	H.	Cl.
	Per ct.	*Per ct.*	*Per ct.*
1	61.34	4.49	1.79
2	61.02	4.45	1.76
Average	61.18	4.47	1.77

If the chlorin were an integral part of the molecule, then the molecule must be an extremely complicated structure with a molecular weight of about 2,000, whereas the phloroglucid of epihydrin aldehyde would be expected to have a molecular weight of less than 200. It seems probable, therefore, that the chlorin is present as an impurity in the form of adsorbed hydrochloric acid, and on this assumption the following revised analytical results have been calculated:

	C.	H.
	Per ct.	*Per ct.*
Found 1	62.48	4.53
2	62.16	4.48
Average	62.32	4.50
$C_{15}H_{12}O_6$	62.49	4.20
$C_{15}H_{14}O_6$	62.05	4.86

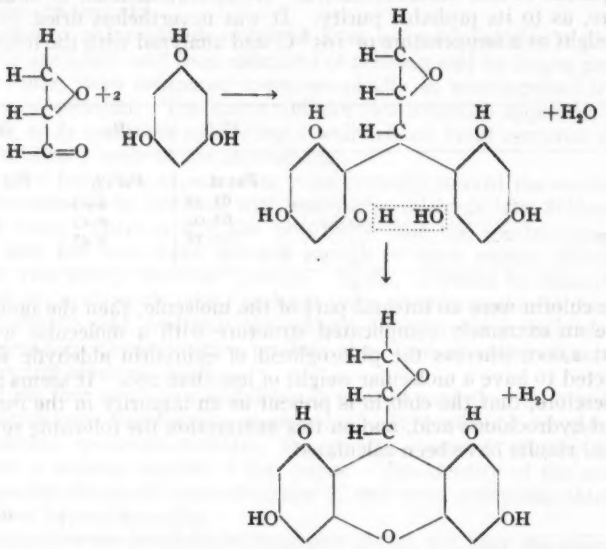
While the revised analytical results correspond somewhat more closely to the formula $C_{15}H_{12}O_6$ than to $C_{15}H_{14}O_6$, they are not sufficiently close to either to indicate a decided choice between them. As a matter of fact they correspond almost exactly to the composition of an equimolecular mixture of the two compounds.

The compound $C_{15}H_{14}O_6$, however, could not very well result from the condensation of epihydrin aldehyde and phloroglucin except through a process of reduction, and according to von Euler (*12*) color formation in similar condensations is generally due to oxidation rather than to reduction. On the other hand, a compound $C_{15}H_4O_6$ would be expected to result from the condensation of acrolein and phloroglucin.

$$CH_2:CH.CHO+2C_6H_3(OH)_3 \rightarrow CH_2:CH.CH:[C_6H_2(OH)_3]_2+H_2O.$$

But whereas some acrolein phloroglucid might well have been formed from uncombined acrolein in the preparation of our analytical material, the acrolein phloroglucid has already been found by experiment to differ in color from the red phloroglucid which is obtained equally well from the acetal of epihydrin aldehyde and from mixtures of acrolein and hydrogen peroxid.

The compound $C_{15}H_{12}O_6$, on the other hand, might easily result from the condensation of epihydrin aldehyde and phloroglucin, through anhydridization of the primary condensation product:



And according to von Euler such anhydrid formation frequently occurs in the condensation reactions of phloroglucin.

Evidently, therefore, the material analyzed was composed principally of the first anhydrid of the phloroglucid originally formed by the condensation of epihydrin aldehyde and phloroglucin, a compound which corresponds to the formula $C_{15}H_{12}O_6$, and which contains the epihydrin aldehyde radical intact. Presumably the anhydrid formation occurs under the dehydrating influence of concentrated hydrochloric acid and is responsible for the change of the originally water-soluble phloroglucid into the extremely insoluble substance that served for our analytical material.

The experiments described in this section have shown (*a*) that substance K is not glyceric aldehyde, dihydroxyaceton, methyl glyoxal, acrolein peroxid, malonicdialdehyde, or oxyacrolein; (*b*) that the colored phloroglucid obtained in the Kreis test from epihydrin aldehyde is identical with the phloroglucids similarly obtained from substance K and from rancid fats; (*c*) that in its reaction with phloroglucin, epihydrin aldehyde enters integrally into the reaction product. It therefore follows that epihydrinaldehyde is formed in the reaction between acrolein and hydrogen peroxid in presence of concentrated hydrochloric acid, and that the color obtained from rancid fats in the Kreis test is due to the formation of a phloroglucid of epihydrin aldehyde.

IS EPIHYDRIN ALDEHYDE A NORMAL CONSTITUENT OF RANCID FATS?

In the following discussion the term "substance R" will be employed to designate the one or more constituents of rancid fats which, either by themselves or together with active oxygen derived from other constituents of the fat, are responsible for the formation of the red color that is obtained from rancid fats in the Kreis test. Substance R might, then, be either epihydrin aldehyde itself, or some other compound or group of compounds, such as acrolein and oleic acid peroxid, that might yield epihydrin aldehyde in presence of concentrated hydrochloric acid. Of these alternative possibilities, the former can not for the present be proved or disproved experimentally, because of our inadequate knowledge of epihydrin aldehyde and its compounds, and our consequent inability to separate and identify them under conditions precluding chemical change. The latter alternative, on the other hand, presents a problem of unknown dimensions, and except for the specific examples mentioned, has not been subjected to extended study.

A number of more or less blind attempts were nevertheless made to separate substance R from rancid fats and rancid oleic acid, in the course of which a number of interesting observations were made.

FRACTIONATION OF RANCID OLEIC ACID.—One hundred cc. of highly rancid oleic acid that gave an intense Kreis test were subjected to steam distillation, 150 cc. of distillate being collected. The distillate gave a negative Kreis test, both in presence and in absence of hydrogen peroxid; but when it was shaken with 25 cc. of ether, a concentration of the ether-soluble material of the distillate being thereby effected, the ether solution gave a distinct, but rather weak, Kreis test. Thus, while some of substance R had distilled, by far the larger amount remained in the undistilled oleic acid.

The undistilled oleic acid, which still gave a strong Kreis test, was shaken with several volumes of hot water. The aqueous extract, when separated, failed to respond to the Kreis test either in presence or absence of hydrogen peroxid; but when it was shaken with a little ether the ether solution gave a weak Kreis test in absence of hydrogen peroxid and a somewhat weaker test in presence of hydrogen peroxid.

After its extraction with water the separated oleic acid, which still gave a strong Kreis test, was dissolved in ether and shaken with concentrated hydrochloric acid. The hydrochloric acid layer, when separated, gave a strong Kreis test of about the same intensity as that obtained from the ethereal layer. When the hydrochloric acid layer was diluted with water and shaken with two separate portions of ether, the ether extract responded to the Kreis test while the extracted hydrochloric acid did not.

By these operations most of the reactive substance had been recovered from the various fractions in ethereal solution. The several ether extracts were combined, dried over calcium chlorid, and allowed to evaporate at room temperature. The following morning a dark-brown oily substance remained. This residue was dissolved in a few cubic centimeters of ether and tested with phloroglucin-hydrochloric acid both with and without previous addition of hydrogen peroxid. The results were at first questionable, but in each case a decided, though not an intense, red color developed after short standing.

Substance R is thus found to be but slightly soluble in water and practically nonvolatile with steam; but it is evidently broken down on

contact with concentrated hydrochloric acid into a substance more soluble in water, or at least in concentrated hydrochloric acid, which is probably identical with substance K.

VACUUM DISTILLATION OF RANCID OLEIC ACID (1).—A considerable quantity of U. S. P. oleic acid was aerated in a series of Folin cylinders in a light window from June 12 to July 26, at the end of which time it was intensely rancid as evidenced by its odor and its strong response to the Kreis test.

One hundred cc. of this material was subjected to fractional distillation at a pressure varying from 0.3 to 2 mm. Identical amounts of the several fractions were used in the tests described.

Fraction 1.—Distilled under 40° C. This fraction consisted of a cloud which could not be condensed.

Fraction 2.—Distilled up to 120° C., but principally between 80° and 110° C. This fraction consisted of 2 to 3 cc. of a pale yellow oil having a slightly scorched and slightly pungent odor. The aldehyde test with Schiff's reagent gave a strong indigo color. The peroxid test with potassium iodid was strongly positive. The Kreis test gave a distinct, though not an intense, red color together with a cream-colored precipitate at the interface between the two layers.

Fraction 3.—Distilled between 120° and 155° C. This fraction was somewhat larger in quantity than Fraction 2, which it resembled in odor and appearance. In the Schiff test a strong purple color was obtained. In the Kreis test it reacted like Fraction 2, but somewhat more weakly. A weak test for peroxids was obtained.

Fraction 4.—Distilled between 155° and 175° C. In physical character this fraction resembled Fractions 2 and 3. Its response to the aldehyde test was weaker than that of the lower fractions, while the Kreis test and the peroxid test were practically negative.

Fraction 5.—Distilled from 175° to 180° C. (2-mm. pressure). This fraction consisted of a pale-yellow oil of bland odor. It gave a faint test for aldehydes, a negative peroxid test, and a negative Kreis test.

Fraction 6.—Distilled up to 185° C. (0.5-mm. pressure). The bulk of the distillate was contained in this fraction, which resembled Fraction 5 in appearance and odor. The Schiff test, the peroxid test, and the Kreis test were all negative.

Fraction 7.—Distilled between 185° and 188° C. About 8 to 10 cc. of distillate were obtained in this fraction, which resembled Fractions 5 and 6 as to its appearance and odor and as to the negative results obtained in the three tests.

Residue.—The residue in the distilling flask was a thick, viscous liquid of a dark, reddish-brown color. It appeared to give negative results in the Schiff test, the peroxid test, and the Kreis test, although in the latter test a weak reaction might have been obscured by the color that the liquid imparted to the hydrochloric acid.

In the foregoing experiment none of the fractions responded to the Kreis test as strongly as the original sample before distillation, and a loss of Substance R is indicated. Since of the fractions collected, Fraction 2 seemed to respond most strongly, the possibility was suggested that even more of Substance R might have been lost in the first, uncondensed portion of the distillate. In the following experiment no such loss could have occurred:

VACUUM DISTILLATION OF RANCID BEEF FAT.—A sample of highly rancid beef fat was distilled at a maximum pressure of 0.5 mm., a trap cooled in liquid air being employed to condense the most volatile portion of the distillate.

Distillation was interrupted at 57° C. for the examination of the distillate. A small amount of material, having a strong odor of heptylic aldehyde, was recovered from the liquid-air tube. When suspended in a little water and tested with phloroglucin-hydrochloric acid a cream-colored precipitate was obtained, but no red color. The same result was obtained when the test was repeated after the previous addition of hydrogen peroxid.

Distillation was then resumed and continued until the water bath in which the distilling flask was immersed had reached the boiling point. A small amount of material, insoluble in water and having the odor of heptylic aldehyde, was again recovered from the liquid-air trap. When dissolved in a little ether and tested with phloroglucin-hydrochloric acid, a cream-colored precipitate and a slightly pink and cloudy hydrochloric acid layer was obtained. When the test was repeated after the previous addition of hydrogen peroxid, a slightly cloudy and yellow hydrochloric acid layer, but no precipitate, was obtained. Both tests were negative for all practical purposes.

The main portion of the distillate had condensed in the main receiver. It gave a weakly positive Kreis test, the color showing the characteristic absorption spectrum.

The undistilled beef fat still gave a strongly positive Kreis test.

VACUUM DISTILLATION OF RANCID OLEIC ACID (II).—In this experiment a specially prepared sample of oleic acid was employed. The method followed in its preparation was essentially that described by Lewkowitsch (*24, p. 52*), except that the barium salt was recrystallized six times from benzene by the method of Farnsteiner instead of from alcohol, and that the recovered oleic acid was further purified by vacuum distillation. In this manner a colorless and odorless product was obtained, which gave a negative test for peroxids and no suggestion of color in the Kreis test. Part of this sample was used for the preparation of oleic acid ozonid, as already described, while the remainder was exposed to the action of light and air. When it had become strongly rancid, as evidenced by its odor and by its response to the Kreis test, it was subjected to vacuum distillation, the distilling flask being connected directly with a receiver immersed in liquid air.

The material in the distilling flask began to boil at room temperature at a pressure of about 0.3 mm., but was heated gradually up to 100° C. before distillation was interrupted. The receiver was found to be covered with a frostlike deposit, and contained in addition a small amount of white fatty solid. On thawing and washing out the receiver with water, the material appeared to be largely, but not entirely, soluble. In the Kreis test the aqueous suspension gave a clear, pale pink color that showed the characteristic absorption band on spectroscopic examination. On repeating the test in presence of hydrogen peroxid the same result was obtained. A positive peroxid test was also obtained. The residue from this distillation seemed to give an undiminished response to the Kreis test and to the peroxid test.

If acrolein were one of a group of compounds forming substance R, it would have distilled completely at a comparatively low temperature and upon addition of hydrogen peroxid the fraction containing the acrolein would have given a strong Kreis test while the undistilled residue would have given a negative test. Substance R appears, rather, to be a compound that is derived from oleic acid, that is practically nonvolatile at pressures of from 0.3 to 2.0 mm. and a temperature of 100° C., and that is gradually decomposed at higher temperatures.

COMPARATIVE SOLUBILITY OF SUBSTANCE R AND EPIHYDRIN ALDEHYDE DIETHYLACETAL.—(*a*) About 25 cc. of melted lard, giving a negative Kreis test, were shaken with two drops of epihydrin aldehyde diethylacetal, which dissolved in the fat almost immediately. The filtered lard reacted intensely in the Kreis test. The odor imparted to the fat was not the characteristic odor of rancidity.

(*b*) The residual lard from the preceding experiment, containing the epihydrin acetal, was shaken with an equal quantity of cold water. After the aqueous extract had separated, it was drawn off and filtered. When 5 cc. of the aqueous extract was treated with a few drops of concentrated hydrochloric acid and a few crystals of phloroglucin, a clear, though not intense, red color was obtained, similar to that obtained from slightly rancid fats in the Kreis test.

(*c*) About 25 cc. of a strongly rancid lard were melted and shaken with about 10 cc. of water. After the aqueous extract had separated, it was drawn off and filtered. When 5 cc. of the extract were treated with a few drops of concentrated hydrochloric acid and a few crystals of phloroglucin, a distinct, but somewhat weak, red color was obtained, which was similar in all respects to that which was obtained in the same manner from the water extract of the sweet lard to which epihydrin aldehyde diethylacetal had been added.

It thus appears that substance R and epihydrin aldehyde diethylacetal are alike in their solubilities in water and in fat, and the possibility is suggested that substance R may be one of the acetals of epihydrin aldehyde.

While it has not been possible to isolate substance R, or to establish its identity, the foregoing experiments have indicated that it is but slightly soluble in water, that it is practically nonvolatile with steam, that it is slightly volatile at pressures of from 0.2 to 0.5 mm. and a temperature of 100° C., and that it is gradually decomposed at higher temperatures, at least in presence of rancid fats. Substance R, therefore, can not contain free acrolein, which is readily volatile at low temperatures and quite soluble in water; presumably it is not free epihydrin aldehyde, which would be expected to resemble acrolein in these respects.

At the same time, substance R resembles epihydrin aldehyde diethylacetal as regards its solubility in fats and in water and in the ability of its dilute aqueous solution to react in the characteristic manner with phloroglucin in the presence of minimal amounts of acid. These facts suggest that substance R may contain the epihydrin aldehyde radical intact, and may be constituted as an acetal, possibly the glyceryl acetal, of epihydrin aldehyde.

SPECTROPHOTOMETRIC OBSERVATIONS

It remains to describe the results of the spectrophotometric color comparisons, by means of which we have established the identity of the phloroglucids obtained from substance K, epihydrin aldehyde diethylacetal, and rancid fats, respectively. For a discussion of the principles underlying the spectrophotometric method, and a description of the instruments employed, the reader is referred to standard text books on the subject. The author is indebted to Dr. H. Wales, of the Bureau of Chemistry, who made the present examinations by means of an instrument that employs rotating nicol prisms for varying the intensity of the normal spectrum with which the absorption spectrum is compared.

The several solutions examined were prepared as follows:

(*a*) *From acrolein-hydrogen peroxid.*—A dilute aqueous solution of acrolein was treated with an excess of hydrogen peroxid, and a few drops of the mixture were treated in a separatory funnel with about 10

cc. of concentrated hydrochloric acid and an equal quantity of a 1 per cent solution of phloroglucin in ether. The amount of acrolein-hydrogen peroxid solution employed was so chosen that the color obtained was of suitable intensity for the spectrophotometric examination. The colored hydrochloric acid layer was drawn off into a glass cell for observation.

(*b*) *From epihydrin aldehyde diethylacetal.*—One drop of epihydrin aldehyde diethylacetal was dissolved in 5 cc. of ether, and varying amounts of this solution were treated as described above until a color of suitable intensity was obtained. The colored hydrochloric acid layer was then drawn off into a glass cell for examination.

(*c*) *From rancid oleic acid.*—About 20 cc. of rancid oleic acid, from a specimen which had been originally of a high degree of purity, were dissolved in ether and treated with about 20 cc. of concentrated hydrochloric acid and a similar quantity of a 1 per cent ethereal solution of phloroglucin. After the development of color, the slightly turbid hydrochloric acid layer was drawn off for examination.

(*d*) *From epihydrin aldehyde diethylacetal.*—One drop of epihydrin aldehyde diethylacetal was dissolved in 5 cc. of ether, and 0.3 cc. of this solution were dissolved in 5 cc. of water. The aqueous solution was then acidified with 10 drops of concentrated hydrochloric acid and treated with a few small crystals of phloroglucin. After about 10 minutes, when the color seemed to have reached its full intensity, the aqueous solution was transferred to a glass cell for observation.

(*e*) *From rancid lard.*—About 20 cc. of rancid lard were melted and shaken with about 10 cc. of water. After separation the water extract was drawn off and filtered, and was then brought to an acidity comparable to that obtaining in the previous experiment and treated with a few small crystals of phloroglucin. After the development of color, the aqueous solution was transferred to a glass cell for spectrophotometric examination.

The results obtained in the spectrophotometric examination of the several solutions are represented graphically in the accompanying charts. Figure 1 compares the spectra of the several concentrated hydrochloric acid solutions, while figure 2 compares the spectra of the weakly acid aqueous solutions. In these graphs the curves are designated by roman capital letters corresponding to the letters designating the solutions just listed, the abscissas represent wave lengths of light, and the ordinates represent the degree of absorption expressed as logarithms of the fraction—

$$\frac{\text{Intensity of incident light of given wave length}}{\text{Intensity of transmitted light of same wave length}}$$

For clear solutions of a pure dyestuff in a given solvent, the height of the absorption curve at any given wave length will be proportional to the concentration of the solution, and will bear a fixed ratio to the height of the same curve at any other given wave length. The curves obtained for all concentrations will therefore exhibit a family resemblance, in that the point of maximum absorption will always correspond to the same wave length, and that the curves will exhibit a degree of likeness in contour. A curve obtained from a more concentrated solution may be scaled down by dividing each ordinate by the same ratio to form a new curve that may be superimposed on a curve obtained from a less concentrated solution of the same dyestuff. Such a similarity of absorption spectra is generally considered to be sufficient to establish the identity

of the dyestuff contained in any two solutions, provided each dyestuff is known to belong to the same class and to be dissolved in the same solvent.

Change of solvent may occasion a change in the location of the point of maximum absorption, or even alter completely the appearance of the absorption curve; while turbidity of the solution will occasion a uniform absorption throughout the length of the spectrum without altering, however, the location of the point of maximum absorption or changing the general aspect of the curve.

Referring to figure 1, it will be observed that some degree of uniform absorption has occurred in case of solution *b*. With due allowance for

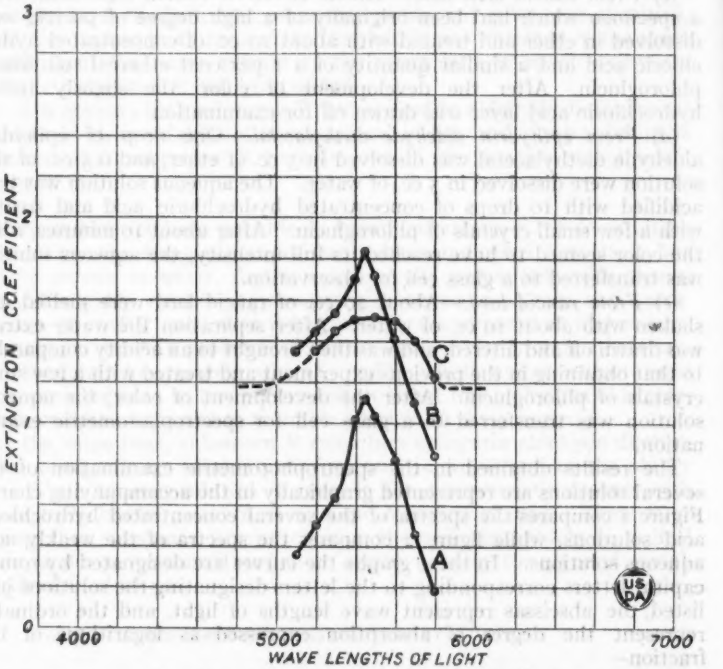


FIG. 1.—Absorption curves of concentrated hydrochloric acid solutions of phloroglucin condensation products obtained from (A) acrolein and hydrogen peroxid, (B) epihydrin aldehyde diethylacetal, and (C) rancid oleic acid.

the slight turbidity of the solution *b*, the curves obtained from solutions *a* and *b* are seen to be practically identical, being similar in contour and exhibiting maximum absorption for the same wave length of light. Inasmuch as each color is known to be a phloroglucid of a carbonyl compound, and to be dissolved in the same solvent, identity of composition is clearly established, proving that the phloroglucid obtained from mixtures of acrolein and hydrogen peroxid is identical with that obtained from epihydrin aldehyde diethylacetal.

In case of solution *c*, the turbidity was so great in proportion to the intensity of color that each reading was subject to a comparatively large experimental error. While the location of the point of maximum ab-

sorption in curve C is slightly different from that in curves A and B, this difference is nevertheless within the limit of experimental error, so that even from this experiment it is extremely probable that the phloroglucid from rancid fats is identical with that obtained from substance K and from epihydrin aldehyde diethylacetal.

The results obtained from the examination of the weakly acid aqueous solutions of the phloroglucids prepared from epihydrin aldehyde diethylacetal and rancid lard, respectively, are plotted graphically in figure 2. The change in solvent was made in the interest of obtaining optically clear solutions and has been accompanied by a slight shift in the location

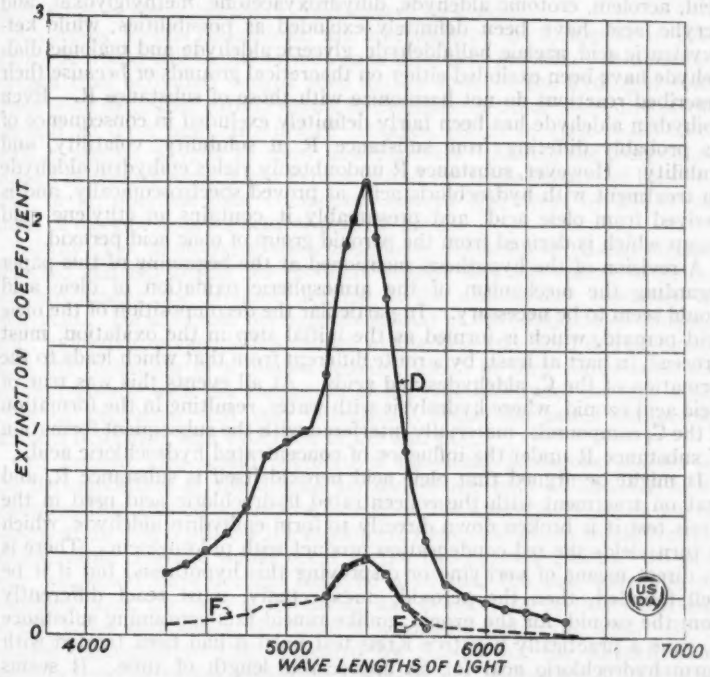


FIG. 2.—Absorption curves of weakly acid aqueous solutions of phloroglucin condensation products obtained from (D) epihydrin aldehyde diethylacetal, and (E) rancid lard. Curve F is obtained by scaling down curve D.

of the point of maximum absorption for the phloroglucid of epihydrin aldehyde. While the location of the points of maximum absorption in curves D and E is identical, the curves indicate a wide difference in intensity of color between the solutions from which they were derived. Curve F was therefore prepared by scaling down curve D, in the manner already described, and is seen to be practically identical in all respects to curve E. Since the colors in both cases are known to belong to the same class of dyestuffs and to be dissolved in the same solvent, this affords final proof, as far as such proof can be obtained, of the identity of the phloroglucids derived from rancid lard and from epihydrin aldehyde, respectively.

DISCUSSION OF RESULTS

The experiments reported in this paper have shown, on the basis of spectrophotometric evidence, that epihydrin aldehyde diethylacetal and the compounds that have been termed substance K and substance R yield identical condensation products with phloroglucin in the Kreis test. They have also identified substance K, formed by interaction of acrolein and hydrogen peroxid in strongly acid solution, as epihydrin aldehyde.

The chief point of interest is the chemical nature of substance R. Most of the saturated acids and aldehydes up to C_9 compounds, together with hydroxystearic acid, dihydroxystearic acid, diketostearic acid, azelaic acid, acrolein, crotonic aldehyde, dihydroxyacetone, methylglyoxal, and acrylic acid have been definitely excluded as possibilities; while ketoxystearic acid, azelaic halfaldehyde, glyceric aldehyde and malonic dialdehyde have been excluded either on theoretical grounds or because their described reactions do not harmonize with those of substance R. Even epihydrin aldehyde has been fairly definitely excluded in consequence of its probably differing from substance R in solubility, volatility, and stability. However, substance R undoubtedly yields epihydrin aldehyde on treatment with hydrochloric acid, as proved spectroscopically, and is derived from oleic acid; and presumably it contains an ethylene oxid group which is derived from the peroxid group of oleic acid peroxid.

A revision of the hypothesis mentioned at the beginning of this paper regarding the mechanism of the atmospheric oxidation of oleic acid would seem to be necessary. In particular the decomposition of the oleic acid peroxid, which is formed as the initial step in the oxidation, must proceed, in part at least, by a route different from that which leads to the formation of the C_9 aldehydes and acids. At all events this was true of oleic acid ozonid, where hydrolysis with water, resulting in the formation of the C_9 compounds, materially interfered with the subsequent formation of substance R under the influence of concentrated hydrochloric acid.

It might be argued that oleic acid peroxid itself is substance R, and that on treatment with the concentrated hydrochloric acid used in the Kreis test it is broken down directly to form epihydrin aldehyde, which in turn yields the red condensation product with phloroglucin. There is no direct means of verifying or disproving this hypothesis; but if it be well founded, then the peroxid, unexpectedly, must react differently from the ozonid, for the ozonid, unlike rancid fats containing substance R, gave a practically negative Kreis test until it had been treated with warm hydrochloric acid for an appreciable length of time. It seems more likely, therefore, that substance R is formed from oleic acid peroxid at a later stage in the atmospheric oxidation of oleic acid.

What, then, are the steps leading to the formation of substance K from oleic acid peroxid, and at what point of the process is substance R formed? It seems unreasonable to suppose that the C_{18} chain should rupture between two saturated carbon atoms while the weaker peroxid or ozonid grouping is left intact. To account for the formation of heptylic aldehyde, and of substance R and eventually of epihydrin aldehyde with its ethylene oxid group, it would seem necessary to suppose that the molecule is first weakened at the point of eventual cleavage by the introduction of new double bonds.

The introduction of new double bonds into the oleic acid chain under the influence of atmospheric oxygen is not exactly an occurrence that would be expected. The substitution of a double bond for a single bond is ordinarily attended with considerable difficulty and, in the laboratory,

generally involves the use of vigorous reagents. Linolenic acid is not readily nor profitably prepared from oleic acid. Yet, in Nature the transition from the more saturated to the less saturated acids, and from the less saturated to the more saturated, must frequently be made (*8*), and cleavage of even the saturated acids into smaller fragments, and eventual decomposition into carbon dioxid and water, must occur.

The idea that the substitution of double bonds for single bonds in the oleic acid chain may be brought about by natural processes has, therefore, nothing intrinsically unreasonable about it; and this is what is believed to occur, possibly only as a subordinate side reaction, at an early stage in the atmospheric oxidation of oleic acid.

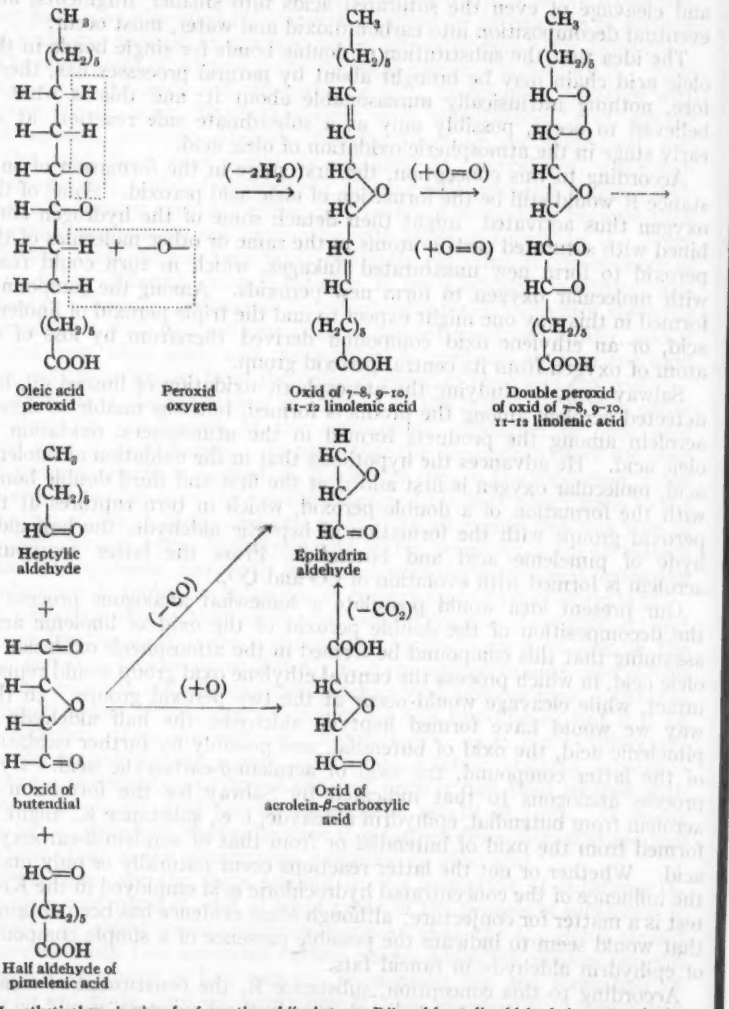
According to this conception, the first stage in the formation of substance R would still be the formation of oleic acid peroxid. Some of the oxygen thus activated might then detach some of the hydrogen combined with saturated carbon atoms in the same or other molecules of the peroxid to form new unsaturated linkages, which in turn could react with molecular oxygen to form new peroxids. Among the compounds formed in this way one might expect to find the triple peroxid of linolenic acid, or an ethylene oxid compound derived therefrom by loss of an atom of oxygen from its central peroxid group.

Salway (*33*), in studying the atmospheric oxidation of linseed oil, has detected acrolein among the products formed, but was unable to detect acrolein among the products formed in the atmospheric oxidation of oleic acid. He advances the hypothesis that in the oxidation of linolenic acid, molecular oxygen is first added at the first and third double bonds with the formation of a double peroxid, which in turn ruptures at the peroxid groups with the formation of heptylic aldehyde, the half aldehyde of pimelenic acid and butendial. From the latter compound acrolein is formed with evolution of CO and CO_2.

Our present idea would postulate a somewhat analogous process in the decomposition of the double peroxid of the oxid of linolenic acid, assuming that this compound be formed in the atmospheric oxidation of oleic acid, in which process the central ethylene oxid group would remain intact, while cleavage would occur at the two peroxid groups. In this way we would have formed heptylic aldehyde, the half aldehyde of pimelenic acid, the oxid of butendial, and possibly by further oxidation of the latter compound, the oxid of acrolein-β-carboxylic acid. By a process analogous to that indicated by Salway for the formation of acrolein from butendial, epihydrin aldehyde, i. e., substance K, might be formed from the oxid of butendial or from that of acrolein-β-carboxylic acid. Whether or not the latter reactions occur naturally or only under the influence of the concentrated hydrochloric acid employed in the Kreis test is a matter for conjecture; although some evidence has been obtained that would seem to indicate the possible presence of a simple compound of epihydrin aldehyde in rancid fats.

According to this conception, substance R, the constituent of rancid fats that is responsible for their behavior in the Kreis test, would be the oxid of a double peroxid of 7–8, 9–10, 11–12 linolenic acid, the oxid of butendial, the oxid of acrolein –β– carboxylic acid, or a simple compound of epihydrin aldehyde. In any one of the latter three cases the Kreis test would have an added significance, inasmuch as the formation of substance R would then involve the simultaneous production of a chemically equivalent amount of heptylic aldehyde, the compound that Scala has held to be responsible for the rancid odor. And in this case a

positive Kreis test, controlled spectroscopically, of course, would give an indirect indication of the presence of heptylic aldehyde, and the intensity of the rancid odor might even be gaged by the intensity of the color obtained.[5]


Oleic acid peroxid
Peroxid oxygen
Oxid of 7–8, 9–10, 11–12 linolenic acid
Double peroxid of oxid of 7–8, 9–10, 11–12 linolenic acid
Heptylic aldehyde
Epihydrin aldehyde
Oxid of butendial
Oxid of acrolein-β-carboxylic acid
Half aldehyde of pimelenic acid

Hypothetical mechanism for formation of "substance R" and heptylic aldehyde by atmospheric oxidation of oleic acid.

[5] Since the submission of the present article for publication, Holm and Greenbank (*10*), exposing butterfat and lard to the action of oxygen at a temperature of 95° C., have found that the intensity of the Kreis test is "directly proportional to the amount of oxygen absorbed by a fat," but that the compounds responsible for the rancid odor do not accumulate in "stoichiometric ratio to the compounds that give the Kreis test." They suggest, however, that the odorous compounds may be destroyed in secondary reactions after their initial formation (oxidation of aldehydes to acids?), and that there is a legitimate question as to whether similar relations would obtain in the spontaneous development of rancidity at usual temperatures. They have privately stated, moreover, that they do not regard their results as being at variance with the hypothesis here proposed regarding the mechanism of the oxidation of oleic acid.

According to the same principle some molecules of oleic acid might be oxidized to compounds containing more than three double bonds, which in turn might give rise to corresponding peroxids and their cleavage products. The hypothesis in this way might be made to account for the presence in rancid fats not only of the C_7 aldehydes and acids but of the whole series of saturated aldehydes and acids found by Scala, as well as for the presence of many other compounds not previously described.

It can at least be said of this hypothesis that it does not appear to be contradicted by such experimental evidence as is available, and that in the absence of contradictory evidence it seems to explain much that is otherwise difficult to account for. Whether or not it can be substantiated by experimental evidence remains to be seen; but it is the author's intention, should his duties permit, to attempt the preparation of the substances, other than the epihydrin aldehyde compounds, that have been suggested to account for the positive Kreis test obtained with rancid fats, and to examine these substances as to their reaction with phloroglucin-hydrochloric acid.

SUMMARY

The results of the experiments recorded in this paper seem to justify a number of more or less definite statements regarding the odor and reactions of rancid fats and the cause of the rancid condition.

1. The odor of heptylic aldehyde in itself and in presence of fresh fats is sufficiently suggestive of the rancid odor to establish the reasonableness of Scala's contention that it is the component of rancid fats that is primarily responsible for their rancid odor.

2. As a result of the direct examination of the following compounds, it appears that none of them contribute appreciably, if at all, toward the rancid odor of fats:

Hydroxystearic acid.	Heptylic acid.	Acrolein.
Dihydroxystearic acid.	Caprylic acid.	Crotonic aldehyde.
Diketostearic acid.	Nonylic acid.	Methylglyoxal.
Formic acid.	Azelaic acid.	Dihydroxyacetone.
Acetic acid.	Formaldehyde.	Acrylic acid.
Butyric acid.	Acetaldehyde.	
Caproic acid.	Butyric aldehyde.	

It also appears, on theoretical grounds, that neither ketoxystearic acid nor azelaic half aldehyde contributes to the odor of rancid fats. Direct examination of nonylic aldehyde, on the other hand, suggests that this compound may be partly responsible for the rancid odor.

3. The typical rancid condition may be reproduced, in all essential respects at least, by the atmospheric oxidation of pure oleic acid, and by suitable treatment of oleic acid ozonid. The glycerin radical and the unsaponifiable matter of natural fats would appear, therefore, not to be necessarily involved in the development of rancidity.

4. As the result of direct examination each of the following compounds has been eliminated as the cause of the Kreis test given by rancid fats:

Hydroxystearic acid.	Caprylic acid.	Nonylic aldehyde.
Dihydroxystearic acid.	Nonylic acid.	Acrolein.
Diketostearic acid.	Azelaic acid.	Crotonic aldehyde.
Formic acid.	Acrylic acid.	Methylglyoxal.
Acetic acid.	Formaldehyde.	Dihydroxyacetone.
Butyric acid.	Acetaldehyde.	Oleic acid ozonid.
Caproic acid.	Butyric aldehyde.	
Heptylic acid.	Heptylic aldehyde.	

On theoretical grounds, oleic acid peroxid, azelaic half aldehyde, and ketoxystearic acid also seem to be eliminated; while glyceric aldehyde is eliminated on the basis of statements in the literature.

5. The color obtained in the Kreis test on rancid fats has been found to be spectroscopically identical with that obtained in the Kreis test on mixtures of acrolein and hydrogen peroxid.

6. The compound that results from the interaction of acrolein and hydrogen peroxid, that responds to the Kreis test, and that has been referred to in this paper as substance K, has been identified as epihydrin aldehyde. It could not be isolated, however, because of its instability, but its diethylacetal has been synthesized.

7. The constituent of rancid fats that causes the Kreis test is not free epihydrin aldehyde, but it is certain that it gives rise to epihydrin aldehyde when the rancid fat containing it is brought into contact with the concentrated hydrochloric acid used in the Kreis test. There is some reason for supposing that substance R may be an acetal of epihydrin aldehyde.

8. A positive reaction in the Kreis test, when the test is performed in the usual manner, is not always a reliable indication of rancidity in fats. A large number of compounds react with phloroglucin-hydrochloric acid to give a red color that, to the unaided eye, is indistinguishable from the color obtained with rancid fats. Such a reaction is notoriously given by many samples of nonrancid cottonseed oil. When the test is modified in such manner that the color obtained is examined spectroscopically, however, the modified test becomes a reliable index of the rancid condition.

9. A mechanism has been provisionally suggested to explain the formation of epihydrin aldehyde or its precursors in the atmospheric oxidation of oleic acid. This mechanism also provides for the formation of heptylic aldehyde and of such other fatty aldehydes as other workers have isolated from rancid fats.

LITERATURE CITED

(1) ARPPE, A. E.
1862. UEBER DIE AZELAÏNSÄURE. *In* Liebig's Ann. Chem. Bd. 124, p. 86–98.

(2) BEILSTEIN, F. K.
1918–20. HANDBUCH DER ORGANISCHEN CHEMIE. Aufl. 4. Bd. 1–2. Berlin.

(3) BERGH, Gustaf Fr.
1909. ÜBER DIE DARSTELLUNG DES ACROLEINS. *In* Jour. Prakt. Chem., N. F., Bd. 79, p. 351–357, illus. Bibliographical footnotes.

(4) BROMEIS, C.
1840. UEBER DIE EINWIRKUNG DER SALPETERSÄURE AUF STEARINSÄURE UND OELSÄURE UND DIE HIERDURCH ERZEUGTEN PRODUKTE. *In* Liebig's Ann. Chem., Bd. 35, p. 86–112.

(5) BROWNE, C. A., Jr.
1899. A CONTRIBUTION TO THE CHEMISTRY OF BUTTER-FAT. III. THE CHEMISTRY OF RANCIDITY IN BUTTER-FAT. *In* Jour. Amer. Chem. Soc., v. 21, p. 975–994. Bibliographical footnotes.

(6) CARETTE, H.
1886. SUR L'OXYDATION DES ACIDES DES GRAISSES. *In* Compt. Rend. Acad. Sci. [Paris], t. 102, p. 692–693.

(7) CLAISEN, L.
1903. ZUR KENNTNISS DES PROPARGYLALDEHYDS UND DES PHENYLPROPARGYLALDEHYDS. *In* Ber. Deut. Chem. Gesell., Jahrg. 36, p. 3664–3673. Bibliographical footnotes.

(8) DUCLAUX, E.
1887. SUR LA MIGRATION DES MATIÈRES GRASSES. *In* Ann. Inst. Pasteur, ann. 1, p. 347–355.

(9) DURRANS, Thomas H.
1919. THE RELATIONSHIP BETWEEN ODOUR AND CHEMICAL CONSTITUTION. *In* Perfumery and Essential Oil Rec., v. 10, p. 104–136.

(10) EMERY, James A., and HENLEY, R. R.
1922. STUDIES ON RANCIDITY. I. THE INFLUENCE OF AIR, LIGHT, AND METALS ON THE DEVELOPMENT OF RANCIDITY. *In* Jour. Indus. and Engin. Chem., v. 14, p. 937–940.
(11) ENGLER, C., and WEISSBERG, J.
1904. KRITISCHE STUDIEN ÜBER DIE VORGÄNGE DER AUTOXYDATION. xi, 204 p. Braunschweig. Bibliographical footnotes.
(12) EULER, Astrid Cleve von.
1921. OM KONDENSATIONER MELLAN KARBONYLFÖRENINGAR OCH RESORCIN (RESP. ORCIN) ELLER FLOROGLUCIN. *In* Ark. Kemi, Mineral. och Geol., Bd. 8, No. 3, 40 p.
(13) FAHRION, W.
1892. ZUR PRÜFUNG VON LEINÖLFIRNISS. *In* Ztschr. Angew. Chem., Jahrg. 1892, p. 171–173.
(14) ———
1893. BEITRÄGE ZUR ANALYSE DER FLÜSSIGEN FETTE. *In* Chem. Ztg., Jahrg. 17, p. 434–436.
(15) FISCHER, Emil, and TAFEL, Julius.
1887. SYNTHETISCHE VERSUCHE IN DER ZUCKERGRUPPE. II. *In* Ber. Deut. Chem. Gesell., Jahrg. 20, p. 3384–3391. Bibliographical footnotes.
(16) HARRIES, C., and TÜRK, H. O.
1907. UEBER DIE SPALTUNGSPRODUCTE DER OELSÄURE-OZONIDE. *In* Ber. Deut. Chem. Gesell., Jahrg. 39, (1906), p. 3732–3737.
(17) HEFFTER, A.
1904. ÜBER DIE ZERLEGUNG DES JODKALIUMS DURCH FETTE. *In* Schweiz. Wchnschr. Chem. u. Pharm., Jahrg. 42, p. 320–322.
(18) HOLDE, D., and MARCUSSON, J.
1903. OXYDATION VON OELSÄURE DURCH PERMANGANAT BEI GEGENWART GERINGER MENGEN ALKALI. *In* Ber. Deut. Chem. Gesell., Jahrg. 36, p. 2657–2662. Bibliographical footnotes.
(19) HOLM, George E., and GREENBANK, George R.
1923. QUANTITATIVE ASPECTS OF THE KREIS TEST. *In* Jour. Indus. and Engin. Chem., v. 15, p. 1051–1053.
(20) KERR, Robert H.
1921. THE CAUSE AND PREVENTION OF RANCIDITY. *In* Cotton Oil Press, v. 5, no. 3, p. 45–48.
(21) ——— and SORBER, D. G.
1923. THE ANALYTICAL DETECTION OF RANCIDITY. *In* Jour. Indus. and Engin. Chem., v. 15, p. 383–385.
(22) KOBERT, Karl.
1907. EIN BEITRAG ZUR PHLOROGLUZIN-SALZÄURE-REAKTION. *In* Ztschr. Analyt. Chem., Jahrg. 46, p. 711–714.
(23) LAURENT, Aug.
1837. RECHERCHES DIVERSES DE CHIMIE ORGANIQUE. PT. 2. ACTION DE L'ACIDE NITRIQUE SUR L'ACIDE OLÉIQUE. *In* Ann. Chim. et Phys., sér. 2, t. 66, p. 154–181.
(24) LEWKOWITSCH, J.
1913. CHEMICAL TECHNOLOGY AND ANALYSIS OF OILS, FATS, AND WAXES. Ed. 5, v. 1, xxii, 668 p., 59 fig. London.
(25) MOUREU, Charles, and DUFRAISSE, Charles.
1922. LES ALTÉRATIONS DE L'ACROLÉINE ET LES ANTIOXYGÈNES. III. *In* Bul. Soc. Chim. France, t. 31, p. 1152–1176, 4 fig. Bibliographical footnotes.
(26) NICOLET, Ben H., and JURIST, Alfred E.
1922. C_{18} FATTY ACIDS. IV. A REARRANGEMENT OF THE BENZILIC ACID TYPE IN THE ALIPHATIC SERIES. *In* Jour. Amer. Chem. Soc., v. 44, p. 1136–1141. Bibliographical footnotes.
(27) ——— and LIDDLE, Leonard M.
1916. THE OCCURRENCE OF AZELAIC ACID AS A PRODUCT OF THE SPONTANEOUS OXIDATION OF FATS. *In* Jour. Indus. and Engin. Chem., v. 8, p. 416–417.
(28) OVERBECK, Otto.
1866. UEBER DIE ABKÖMMLINGE DER OELSÄURE. *In* Liebig's Ann. Chem., Bd. 140, p. 39–75.
(29) PASTUREAU and LAUNAY, Ch.
1919. SUR LE GLYCOL CÉTONIQUE DÉRIVÉ DE L'OXYDE DE MÉSITYLE. *In* Bul. Soc. Chim. France, sér. 4, t. 25, p. 543–544, 593–597.

(30) PINKUS, Georg.
1898. UEBER DIE EINWIRKUNG VON BENZHYDRAZID AUF GLUCOSE. *In* Ber. Deut. Chem. Gesell., Jahrg. 31, p. 31–37. Bibliographical footnotes.
(31) REDTENBACHER, J.
1846. UEBER EINE ALLGEMEINE ENTSTEHUNGSWEISE DER SÄUREN $(CH)_N O_4$ MIT DEM SIEDPUNKT UNTER 300° C. *In* Liebig's Ann. Chem., Bd. 59, p. 41–57.
(32) RITSERT, Ed.
1890. UNTERSUCHUNGEN ÜBER DAS RANZIGWERDEN DER FETTE. *In* Naturw. Wchnschr., Bd. 5, p. 331–334, 343–346, 354–356, 364–366, 374.
(33) SALWAY, Arthur Henry.
1916. STUDIES ON THE OXIDATION OF UNSATURATED FATTY OILS AND UNSATURATED FATTY ACIDS. PART I. THE FORMATION OF ACROLEIN BY THE OXIDATION OF LINSEED OIL AND LINOLENIC ACID. *In* Jour. Chem. Soc. [London] Trans., v. 109, pt. 1, p. 138–145.
(34) SAYTZEFF, Alexander.
1886. UEBER DIE OXYDATION DER OEL- UND ELAÏDINSÄURE MIT KALIUMPERMANGANAT IN ALKALISCHER LÖSUNG. *In* Jour. Prakt. Chem., N. F., Bd. 33, p. 300–318.
(35) SAYTZEFF, Michael; SAYTZEFF, Constantin; and SAYTZEFF, Alexander.
1887. ZUR GESCHICHTE DER OXYSTEARINSÄUREN VERSCHIEDENEN URSPRUNGS. *In* Jour. Prakt. Chem., N. F., Bd. 35, p. 369–390. Bibliographical footnotes.
(36) SCALA, Alberto.
1897. IRRANCIDIMENTO E RANCIDITÀ DEI GRASSI. *In* Staz. Sper. Agr. Ital., v. 30, p. 613–630.
(37) ———
1908. SU DI ALCUNE SOSTANZE VOLATILI E NON VOLATILI CHE SI FORMANO NELL'IRRANCIDIMENTO DEI GRASSI. *In* Gaz. Chim. Ital., v. 38, p. 307–327.
(38) THIEME, Carl.
1905. UEBER DIE ISOMERIE DER OEL- UND ELAÏDINSÄURE. *In* Liebig's Ann. Chem., Bd. 343, p. 354–360. (Harries, C. Ueber die Einwirkung des Ozons auf organische Verbindungen. Experimenteller Theil II.)
(39) VINTILESCO, J., and POPESCO, Alin.
1915. SUR UNE RÉACTION BIOCHIMIQUE DES GRAISSES RANCES. *In* Jour. Pharm. et Chim., t. 12, p. 318–323. Bibliographical footnotes.
(40) WALKER, Herbert S.
1906. THE KEEPING QUALITIES AND THE CAUSES OF RANCIDITY IN COCONUT OIL. *In* Philippine Jour. Sci., v. 1, p. 117–142.
(41) WINCKEL, Max.
1905. UEBER DIE ZERSETZUNG DER FETTE UND DIE URSACHE DES RANZIGWERDENS DERSELBEN. *In* Apoth. Ztg., Jahrg. 20, p. 690–692. Bibliographical footnotes.
(42) WITZEMANN, Edgar J.
1914. THE SYNTHETIC PREPARATION OF *dl*-GLYCERIC ALDEHYDE. *In* Jour. Amer. Chem. Soc., v. 36, p. 1908–1916. Bibliographical footnotes.
(43) WOHL, A.
1898. UEBER DIE ACETALE DES ACROLEINS UND DES GLYCERINALDEHYDS. *In* Ber. Deut. Chem. Gesell., Jahrg. 31, p. 1796–1801. Bibliographical footnotes.
(44) ——— and MOMBER, Fr.
1915. ÜBER *d*- UND *l*- GLYCERINALDEHYD. *In* Ber. Deut. Chem. Gesell., Jahrg. 47 (1914), p. 3346–3358.
(45) ——— and NEUBERG, C.
1900. ZUR KENNTNISS DES GLYCERINALDEHYDS. *In* Ber. Deut. Chem. Gesell., Jahrg. 33, p. 3095–3110. Bibliographical footnotes.
(46) ——— and SCHWEITZER, H.
1907. ÜBER DEN AMIDOMILCHSÄUREALDEHYD. *In* Ber. Deut. Chem. Gesell., Jahrg. 40, p. 92–102. Bibliographical footnotes.

SOME PHYSIOLOGICAL VARIATIONS IN STRAINS [1] OF RHIZOPUS NIGRICANS [2]

By L. L. HARTER and J. L. WEIMER, *Pathologists, Office of Cotton, Truck and Forage Crop Disease Investigations, Bureau of Plant Industry, United States Department of Agriculture*

Several investigators have demonstrated that *Rhizopus nigricans* Ehrnb. causes a soft rot of the sweet potato (*Ipomoea batatas* Lam.). Recent investigations (*2*)[3] have shown that this organism when isolated from the sweet potato will cause decay of a number of different fruits and vegetables. Likewise an organism has been isolated many times from other crops which appears to be identical with the one associated with the sweet potato soft rot. The decay of a number of fruits and vegetables has frequently been attributed to *Rhizopus nigricans* by other investigators. In view of these investigations and the more recent ones by Lauritzen and Harter (*5*), there appears to be no doubt that *Rhizopus nigricans* is the principal cause of the sweet potato soft rot and of a similar decay of many fruits and vegetables. So far as the writers are aware, no one has attempted to study the physiological similarity or dissimilarity of the different isolations or strains of the organism which is taxonomically called *Rhizopus nigricans;* that is, of the strains of *R. nigricans* isolated from various hosts. Being conscious of the fact that it would be impossible to make a detailed investigation of any considerable number of the possible physiological relations of these strains, the writers decided to study (1) their parasitism, (2) the influence of temperature on their spore germination and subsequent growth, (3) their ability to produce pectinase, and (4), their influence on the hydrogen-ion concentration of the substrate.

This organism has been reported from various parts of the world, hence the question naturally arises as to whether or not *R. nigricans* from Cuba, for example, differs parasitically and physiologically from *R. nigricans* from Maine. The problem, then, in part at least, is concerned in a study of the physiological specialization within the species. Matsumoto (*6*) found that different isolations of *Rhizoctonia solani* varied considerably physiologically, and La Rue and Bartlett (*4*) concluded that by a sufficiently refined technique a nominal species such as *Pestalozzia guepini* might be resolved into an indefinite number of demonstrably distinct strains, the number depending only upon the precision of the methods.

Out of a large number of different strains in the writers' possession, eighteen were selected for study, which were obtained from a variety of hosts widely separated taxonomically. Care was also exercised to select those which were obtained from hosts widely separated geographically, in which it was hoped that any influence of climate might be detected. The isolation key number, the host, the locality, and the name of the person who made the isolation are given for each of the eighteen strains employed in these investigations, in Table I.

[1] Strain as here used does not imply any difference morphologically, but different isolations of the same species.

[2] Accepted for publication Aug. 16, 1923.

[3] Reference is made by number (italic) to "Literature cited," p. 371.

TABLE I.—*Isolation, key number, the host, the locality, and the name of the person who isolated each of the 18 strains of Rhizopus nigricans studied*

Number.	Host.	Locality.	Isolated by—
5061	*Artocarpus integrifolia*...	Soledad, Cuba.....	L. L. Harter.
5053	Cabbage.....	Chicago, Ill.....	G. B. Ramsey.
5063	Unknown.....	Amsterdam, Holland....	Unknown.
5051	Bean.....	Michigan.....	E. A. Bessey.
5054	Strawberry.....	Lewiston, Me.....	Neil E. Stevens.
5056	Pepper.....	Chicago, Ill.....	G. B. Ramsey.
5057	Strawberry.....	Plant City, Fla.....	Neil E. Stevens.
5055	*Anona reticulata*.....	Santiago de las Vegas, Cuba.	S. C. Bruner.
4652	Sweet potato.....	Washington, D. C.....	L. L. Harter.
5052	Unknown.....	Amsterdam, Holland...	Unknown.
5060	*Artocarpus integrifolia*...	Santiago de las Vegas, Cuba.	S. C. Bruner.
5058	*Anona squamosa*.....	.....do.....	S. C. Bruner.
4682	Raspberry	New York, N. Y.....	E. D. Eddy.
4684	Strawberry.....	El Monte, Calif.....	Neil E. Stevens.
4887	*Psidium guajava* L.....	Santiago de las Vegas, Cuba.	S. C. Bruner.
5062	*Musa paradisiaca*.....	Havana, Cuba.....	S. C. Bruner.
5059	Tomato.....	Chicago, Ill.....	G. B. Ramsey.
5050	Peach.....	Washington, D. C.....	J. S. Cooley.

Numbers 5063 and 5052 are plus and minus strains respectively, and were obtained through the Centraalbureau voor Schimmelcultures, Amsterdam, Holland.

Before these strains were employed in these investigations each was studied (not critically) and found to conform to what the writers have identified as *Rhizopus nigricans*. No difference morphologically was detected between any of them.

In view of the fact that more than one species of *Rhizopus* is frequently isolated from a host, each of the strains employed in these investigations was "pure lined," that is, a culture was obtained from a single spore.

The fungi were kept in a vigorous state of growth by frequent transfers to some medium on which they grew well. Sweet potato agar in 100 cc. Erlenmeyer flasks was generally employed. The cultures were held at a temperature of from 20° to 22° C.

PARASITISM

The parasitism of the eighteen strains was tested on sweet potatoes according to the method usually employed by the authors, which is briefly as follows: The potatoes were carefully washed but not disinfected. The inoculations were made by the "well" method (*3*). The potatoes were confined in moist chambers with filter paper in the bottom, and incubated in the dark at a temperature of from 20° to 22° C. The cultures used in making the inoculations were grown in sweet potato decoction, in one experiment for two days and in another for three days. The above temperatures were employed because it had been shown that the strains of *R. nigricans* previously studied grew well at about 20° to 22° C.

At the end of three days, notes were taken of the percentage of infection and the approximate amount of decay. When the potatoes were almost decayed, usually at the end of five days, they were removed from the incubator and final notes taken of the percentage of infection and the total amount of decay. An isolation was made from each infected potato and the organism identified.

The results of these inoculations need not be given in detail. They show, however, that all of the organisms are parasitic, the percentage of infection varying from 80 to 100 per cent. Uninoculated controls did not become infected. Nearly all the infected potatoes were completely decayed at the end of five days when the experiment was terminated. The results of the isolations showed that *R. nigricans* was obtained from every one of the infected potatoes.

These investigations show that *Rhizopus nigricans* from a variety of hosts widely separated geographically is parasitic on sweet potatoes. Although there is some difference in the percentage of infection, this difference is probably no greater than would be expected if the same organism was employed in different sets of experiments. The conclusion drawn is that there is no material difference in the parasitism of any of these strains. A repetition of these experiments gave similar results.

RELATION OF TEMPERATURE TO GERMINATION OF SPORES AND SUBSEQUENT GROWTH

It has been shown above that all the strains of *R. nigricans* are parasitic. It has further been shown that although certain so-called species (*tritici*, *nodosus*, *oryzae*, *delemar*) of *Rhizopus* are very similar morphologically, they are dissimilar physiologically (*8*). In view of these facts, it was proposed to make an investigation to determine whether or not different isolations of *R. nigricans* behave differently physiologically.

The hanging drop method used for studying spore germination was selected from among others tried, as being the one likely to give the most reliable data. Sweet potato decoction was employed as a substrate. Care was taken that each hanging drop contained an approximately equal number of spores. Seven incubators were employed, the temperatures of which are shown in Table II. One hanging drop of each organism was placed in each of the incubators and examined frequently for spore germination. By using all the organisms at one time in each of the incubators, variations due to fluctuations in temperature were largely obviated. These tests were duplicated and the average figures obtained are given in Table II.

When it was desirable to examine the spores, the hanging drops were removed from the incubator and placed under a microscope near by, every effort being made to examine them as quickly as possible. The slide was not out of the incubator usually more than one minute. Spore germination was considered to have taken place when the length of the germ tube of the first germinating spores was equal to the diameter of the spore.

The same incubators were employed in determining the minimum, optimum, and maximum temperatures for growth. The same general methods were employed also with such modifications as were necessary to meet the requirements of the experiments. All the strains were

used at one time in each of the seven incubators, in order to eliminate variations due to fluctuations in the temperatures of the incubators. The organisms were grown on Irish potato agar in Petri dishes. A small drop of a spore suspension was placed in the center of the dish by means of a platinum loop. At the end of twenty-four hours and as often thereafter as desirable the diameter of the growth was measured. The plate was usually entirely covered at the end of forty-eight hours at the optimum temperature. The optimum temperature was considered to be the one at which the entire plate was covered in the fewest number of hours; the maximum, that at which spore germination and growth would just take place. It was more difficult to establish a minimum temperature, since the time element played such an important part. It was found that at some of the lower temperatures germination took place after about 30 days. Usually this was about as long a time as was allowed for germination. However, it is not unlikely that in a longer time germination might have taken place at a lower temperature.

Table II shows in detail the results of the germination tests. Perhaps in no case has the minimum been established. The spores of five organisms germinate at 1.8° C. in from five to seventeen days. It is not unlikely if sufficient time had been allowed the spores of other strains would have germinated at the same or even at a lower temperature. Nevertheless, they had not done so by the end of thirty days. Three hours at a temperature of 31° C. was found to be the average time required to germinate the spores. Therefore, according to the criteria established by the writers, a temperature of 31° C. is the optimum for germination of the spores of the different strains of *R. nigricans*. At temperatures both above and below 31° C. the time required to germinate the spores is increased. At a temperature of 32.5° C. it is increased 60 minutes and at 33.5° C. 1 hour and 37 minutes. No germination took place at 35.5° C., a temperature only four degrees higher than the optimum, which shows that only a slight rise in temperature above the optimum is very injurious to the spores. At temperatures below the optimum the time required to germinate the spores increases much more gradually. If a curve was constructed to show the average time required to germinate the spores at the different temperatures, it would be found that the ascent from the lowest temperatures to the optimum is quite gradual. Above the optimum temperature the curve would fall quite abruptly to zero at 35.5° C. This is the type of curve usually obtained from the results of experiments of this kind. The authors (*8*) have obtained such curves for several different species of this genus.

It is of interest to note some of the variations between the different strains at the same temperature. For example, at 31° C., which is the optimum for all of the organisms with the exception of 4652, there is a variation of from 2 hours and 41 minutes to 4 hours and 37 minutes; the spores of the majority of the strains, however, germinated in less than 3 hours. One strain, 4652, requires special consideration. This organism has been used by the writers in a large number of experiments of various kinds. It was used by them (*8*) when a comparative study was made of the minimum, optimum, and maximum temperatures for spore germination and mycelial growth of the different species of *Rhizopus*. It has also been used for the study of pectinase secretion (*1*), hydrogen-ion concentrations, etc. It was found to be an especially low temperature form and to secrete a small amount of pectinase. It will be noted from an

TABLE II.—*Length of time in hours, minutes, or days required for the spores of the different strains of Rhizopus nigricans to germinate at different temperatures*

Organism No.	Temperature (°C.).																					
	1·8		7·7		15·4		18·9		21·4		25·2		26·7		31·0		32·5		33·5		35·5	
	Days.		*Hrs.*	*Min.*	*Hrs.*	*Min.*	*Hrs.*	*Min.*	*Hrs.*	*Min.*	*Hrs.*	*Min.*	*Hrs.*	*Min.*	*Hrs.*	*Min.*	*Hrs.*	*Min.*	*Hrs.*	*Min.*	*Hrs.*	*Min.*
5061	.....		32	15	6	25	5	5	4	37	3	58	3	40	2	48	3	38	4	41	.....	
5053	.....		36	15	7	45	6	5	5	35	4	23	3	55	2	54	3	32	4	39	.....	
5063	.....		31	45	6	25	5	5	4	32	3	58	3	18	2	48	3	56	4	41	.....	
5051	.....		35	30	6	17	5	5	4	42	4	6	3	27	2	42	3	42	4	51	.....	
5054	.....		41	0	7	22	5	52	5	0	4	14	3	48	2	54	4	1	4	49	.....	
5056	.....	11	33	0	8	10	5	23	4	55	3	58	3	40	3	18	4	7	5	20	.....	
5057	.....	10	48	0	8	27	6	55	6	7	5	42	4	45	4	37	5	30	6	0	.....	
5055	.....		32	30	6	30	5	5	4	32	4	7	3	44	2	41	3	32	4	45	.....	
4652	.....	5	28	0	5	10	4	25	3	55	3	17	2	50	2	54	0	0	0	0	.....	
5052	.....		48	0	8	12	5	37	5	22	4	22	3	41	3	12	3	47	4	50	.....	
5060	.....		46	0	7	35	6	22	5	30	4	18	3	52	3	8	3	51	5	22	.....	
5058	.....		42	0	6	27	5	10	4	40	4	6	3	32	2	50	3	40	4	31	.....	
4682	.....		48	0	7	50	6	25	5	55	4	17	3	25	2	54	3	52	4	55	.....	
4684	.....		45	0	8	25	6	32	5	30	4	40	4	1	2	48	3	46	4	35	.....	
4887	.....		36	15	6	35	5	21	4	36	4	6	3	40	2	45	3	34	4	36	.....	
5062	.....		45	0	8	15	6	0	5	30	4	42	4	1	2	54	3	55	5	2	.....	
5059	.....		35	20	7	10	6	15	5	27	4	28	3	44	2	54	3	39	4	57	.....	
5050	.....	17	34	45	6	35	5	27	4	52	4	6	3	46	3	7	3	55	4	28	.....	
Average	.....		38	40	7	12	5	40	5	5	4	16	3	42	3	0	3	40	4	37	.....	

examination of Table II that it is the lowest temperature form of the 18 strains of *Rhizopus nigricans* studied, its optimum temperature being 26.7° C. If intermediate temperatures had been employed, the optimum would doubtless be somewhat higher. This organism germinated in a shorter length of time (5 days) at 1.8° C. than any of the others tried. As a matter of fact, the spores of this organism germinated in a shorter length of time at all temperatures, except at 31° C., than any of the others. Quite contrary to the results obtained with the other organisms, the spores of 4652 did not germinate at 32.5° and 33.5° C., showing that a temperature much above 31° C. is fatal to them.

TABLE III.—*Diameter (in mms.) of the mycelial disks formed by 18 different strains of Rhizopus nigricans at different temperatures*

Organism No.	7° C. 18 days.	9° C. 7 days.	22.5° C. 1 day.	26.2° C. 1 day.	27° C. 1 day.	30° C. 1 day.	31° C. 5 days.	35° C. 5 days.
5061	0	45	29.5	37.5	39.0	17.1	0	0
5053	0	70	32.0	38.0	39.0	17.9	0	0
5063	0	.....	25.0	35.0	35.0	17.8	10	0
5051	0	70	19.5	24.5	26.0	18.2	0	0
5054	0	.....	27.5	38.5	44.0	16.3	4	0
5056	0	70	30.0	35.5	38.0	13.8	0	0
5057	0	60	20.0	34.0	36.0	12.3	0	0
5055	0	30	31.5	38.5	39.0	20.1	11.2	0
4652	40	25	19.0	26.5	22.0	0	0	0
5052	10	70	24.5	36.5	39.0	14.0	0	0
5060	0	0	29.5	39.0	39.0	18.4	4.2	0
5058	0	6	30.0	38.0	41.0	16.0	17.6	0
4682	32	67	22.5	29.5	32.5	11.0	8.5	0
4684	0	45	31.5	41.0	42.0	14.6	0	0
4887	0	50	23.0	37.0	39.0	21.1	0	0
5062	0	55	30.0	43.0	43.5	22.6	0	0
5059	0	8	29.0	38.0	37.5	19.5	8	0

An examination of Table III shows that the average optimum temperature for the growth of the mycelium of the different strains of *R. nigricans* is about 27° C. There is a considerable falling off in the diameter of the disks at 30° C., only three degrees above the optimum, and at 31° C. only a part of the organisms grew. A reference to Table II will show that this was the optimum temperature for the germination of the spores. The growth curve is in general very similar to the curve of spore germination, the optimum and maximum temperatures for growth, however, being somewhat lower than those for spore germination. In these experiments the spores often germinated at temperatures at which further growth did not take place.

It is interesting to note the results obtained with the organism 4652 with which so much previous work in other connections has been done. It was found in the spore germination tests that this fungus had a lower optimum and maximum than any of the other strains studied. From Table III it is seen that the optimum for its growth is somewhat lower than that for the other fungi, being approximately 26.2° C. At 30° C. and above it made no growth though the spores germinated very well at 31° C. This organism also made a fair growth at a temperature of 7° C., while most of the other strains made no growth at the same temperature.

The conclusions to be drawn from these experiments are, first, that there is a considerable uniformity in the cardinal temperatures for the growth of the mycelium of these different strains; and second, that the optimum and maximum temperatures for growth are several degrees lower than those for spore germination. The authors (*8*) obtained similar results in experiments in which the cardinal temperatures for germination and growth of different species of Rhizopus were studied. It is quite evident from the results obtained in these experiments and elsewhere, as already cited, that the temperature at which the spores will germinate can not be assumed to be that at which infection will take place. As a matter of fact, the results suggest that infection and decay would occur only at temperatures somewhat lower than those required for the germination of the spores.

PECTINASE PRODUCTION AND HYDROGEN-ION CONCENTRATION

Previous investigations showed that several species of *Rhizopus* produced pectinase (*1*) in considerable abundance. In the same experiments it was found, however, that *Rhizopus nigricans* produced none or a very small amount of the macerating principle. This was surprising, in view of the fact that this species was the one responsible for most of the loss of sweet potatoes in storage due to soft rot. It has been noted also that when sweet potatoes are decayed by the organism, both in the laboratory and in commercial storage houses, the cells are separated along the line of the middle lamellae just as they are when decayed by species which produce an abundance of the macerating enzym. Investigations (*7*) have shown that one strain of *R. nigricans*, number 4652, when grown in sweet potato decoction tends to make the substrate less instead of more acid, as is the case of those species which produce a very active macerating principle. Furthermore, it has been found that the expressed juice from sweet potatoes decayed by *R. nigricans* was less acid than that from potatoes decayed by *R. tritici;* also that this juice did not macerate raw disks as readily as the juice from potatoes rotted by *R. tritici*. If, however, the juice of potatoes rotted by *R. nigricans* was made as acid as that from potatoes decayed by *R. tritici*, it macerated sweet-potato disks in the same length of time. In view of these facts, it was proposed to determine if the inability of *R. nigricans* to produce pectinase under cultural conditions as readily as do some of the other species was peculiar to a single strain or characteristic of the species in general, as represented by eighteen different strains, and also whether or not the same peculiarity is true with respect to the acid production.

The method employed in these experiments was as follows: Seventy-five cubic centimeters of sweet potato decoction were placed in 250-cc. Erlenmeyer flasks. Enough flasks were prepared so that duplicate cultures of all the strains could be grown at the same time, thus subjecting all the organisms to as nearly the same conditions as possible. After inoculation the flasks were held in an incubator room with a temperature which varied from 23° to 24° C. At the end of the growth period (four days) the contents of the flasks on which the same strain had grown were made into a compound sample and the hydrogen-ion concentration determined. Raw sweet-potato disks were placed in one portion of the solution in order to determine whether or not pectinase

had been secreted, and, if so, to obtain an idea of the amount as indicated by the rate at which it caused maceration. Control flasks were carried in all the experiments. The rate of maceration in the steamed controls (steamed 15 minutes) of the solutions on which the organisms grew was compared with that in the solutions not steamed.

With respect to pectinase production it may be stated that no maceration of raw sweet-potato disks occurred in any of the solutions at the end of forty-eight hours. There was no apparent difference between the steamed and unsteamed solutions, which seemed to indicate that no pectinase was secreted. The fact that the eighteen organisms all acted alike in this respect seemed to suggest that under these conditions *R. nigricans* characteristically produces none, or at least only a very small amount of pectinase.

The hydrogen-ion concentration of the solution after the organisms had grown on it for four days is shown in Table IV.

TABLE IV.—*Hydrogen-ion concentrations of the sweet-potato decoction after the different strains of R. nigricans had grown on it for 4 days (average of several experiments).*

Organism No.	P_H	Organism No.	P_H	Organism No.	P_H
5061	6.853	5055	6.509	4684	6.560
5053	6.997	4652	6.157	4787	6.718
5063	6.873	5052	5.322	5062	7.021
5054	6.780	5060	4.468	5059	6.836
5056	6.353	5058	6.661	Control	5.158
5057	5.334	4682	5.814		

Table IV shows that all the different strains, with the exception of 5060, decreased the acidity of the solution. In the case of 5060 the acidity of the substrate was considerably increased by the growth of the fungus.

The results of these investigations show that pectinase is not generally produced when a nutrient solution, such as sweet-potato decoction, is used as a substrate.

Just why this is so, is not clear. Results previously published (*1*) show that one strain of *Rhizopus nigricans* (4652) produced a small amount of pectinase when compared with some of the other species. It is quite evident from the results here that the failure to do so is not peculiar to any one strain, but is probably characteristic of the species as a whole. The writers found that, if *R. nigricans* and *R. tritici* were inoculated into sweet potatoes, decay took place in both cases, the cells being separated along the line of the middle lamellae. If raw sweet-potato disks were suspended in the expressed juice, they were macerated in a shorter time in that from potatoes decayed by *R. tritici* than in that decayed by *R. nigricans*. The juice from *R. tritici* decayed potatoes was the most acid. If, however, the expressed juice from *R. nigricans* decayed potatoes was made as acid as the *R. tritici* juice, maceration took place in the same length of time in both cases. It would seem, therefore, that the composition of the substrate has much to do with the production of pectinase. There is probably something lacking in all of the artificial culture media used which is required to stimulate the secretion of the enzyme.

SUMMARY

1. Eighteen strains of *Rhizopus nigricans* have been studied. The organisms used were isolated from hosts widely separated botanically. They were obtained from various parts of the United States and from Europe.

2. They were all found to be about equally parasitic on sweet potatoes.

3. The optimum temperature for the germination of the spores was about 31° C., with the exception of one organism (4652) which had an optimum somewhat lower. The maximum was between 33.5° and 35.5° C., with the exception noted above, which had a maximum close to 31° C. The spores of some of the organisms germinated at 1.8° C.

4. The optimum temperature for mycelial growth was about 26.2° C. and the maximum near to 31° C., with the exception of strain 4652, in which case both the optimum and maximum temperatures were slightly lower. The results show that the spores will germinate at a temperature several degrees higher than that at which the mycelium will grow.

5. None of the strains produced a cell-wall-destroying enzym (pectinase), at least in sufficient quantity to be detected by the methods used when grown on sweet-potato decoction.

6. With one exception, the acidity of the substrate (sweet-potato decoction) was decreased.

LITERATURE CITED

(1) HARTER, L. L., and WEIMER, J. L.
1921. A COMPARISON OF THE PECTINASE PRODUCED BY DIFFERENT SPECIES OF RHIZOPUS. *In* Jour. Agr. Research, v. 22, p. 371–377, 2 fig.

(2) ——— ———.
1922. DECAY OF VARIOUS VEGETABLES AND FRUITS BY DIFFERENT SPECIES OF RHIZOPUS. *In* Phytopathology, v. 12, p. 205–212. Literature cited, p. 212.

(3) ——— ——— and ADAMS, J. M. R.
1918. SWEET-POTATO STORAGE-ROTS. *In* Jour. Agr. Research, v. 15, p. 337–368, pl. 21–27. Literature cited, p. 366–368.

(4) LA RUE, CARL D., and BARTLETT, H. H.
1922. A DEMONSTRATION OF NUMEROUS DISTINCT STRAINS WITHIN THE NOMINAL SPECIES PESTALOZZIA GUEPINI DESM. *In* Amer. Jour. Bot., v. 9, p. 79–92. References, p. 92.

(5) LAURITZEN, J. I., and HARTER, L. L.
1923. SPECIES OF RHIZOPUS RESPONSIBLE FOR THE DECAY OF SWEET POTATOES IN THE STORAGE HOUSE AND AT DIFFERENT TEMPERATURES IN INFECTION CHAMBERS. *In* Jour. Agr. Research, v. 24, p. 441–456, 1 fig. Literature cited, p. 456.

(6) MATSUMOTO, TAKASHI.
1921. STUDIES IN THE PHYSIOLOGY OF THE FUNGI. XII. PHYSIOLOGICAL SPECIALIZATION IN RHIZOCTONIA SOLANI KÜHN. *In* Ann. Mo. Bot. Gard., v. 8, p. 1–62, 6 fig. Bibliography, p. 58–62.

(7) WEIMER, J. L., and HARTER, L. L.
1923. HYDROGEN-ION CHANGES INDUCED BY SPECIES OF RHIZOPUS AND BY BOTRYTIS CINEREA. *In* Jour Agr. Research, v. 25, p. 155–164. Literature cited, p. 163–164.

(8) ——— ———.
1923. TEMPERATURE RELATIONS OF ELEVEN SPECIES OF RHIZOPUS. *In* Jour. Agr. Research, v. 24, p. 1–40, 23 fig. Literature cited, p. 39–40.

60379—24——4

PREPARATION AND PROPERTIES OF COLLOIDAL ARSENATE OF LEAD[1]

By F. J. BRINLEY, *Assistant Entomologist, Fruit Insect Investigations, Bureau of Entomology, United States Department of Agriculture*

INTRODUCTION

During the early work on the control of the Japanese beetle (*Popillia japonica* Newm.), commercial arsenate of lead apparently had a decidedly repellent effect upon the insect. For this reason experiments were undertaken by B. R. Leach and the writer to determine whether the repellent action was due to the physical properties of the arsenate or to some other factor. Among the series of experiments made to determine this point the writer made a study of the preparation of colloidal lead arsenate. The work reported herein was performed by the writer at the Japanese beetle laboratory at Riverton, N. J., during the season of 1920–21.

CHEMICALS USED

An arsenate of lead composed of very fine particles may be prepared by the chemical action of lead nitrate with disodium arsenate. However, when these chemicals react in the presence of a protective colloid, such as gelatin, a colloidal arsenate of lead may be formed.

The chemicals used for the preparation of colloidal lead arsenate are: Lead nitrate ($Pb(NO_3)_2$), disodium arsenate ($Na_2HAsO_4 7H_2O$), and sheet gelatin. According to Haywood and McDonnell [2] the chemical reaction between lead nitrate and disodium arsenate largely results in the formation of acid lead arsenate ($PbHAsO_4$):

$$Pb(NO_3)_2 + Na_2HAsO_4 = PbHAsO_4 + 2NaNO_3.$$

In all probability the gelatin does not enter into the chemical action, but is adsorbed by the particles of lead arsenate as they are formed and seems to prevent the individual particles from uniting and forming larger ones.

Other colloids, such as gum arabic, dextrin, and potato starch, were used but did not prove as satisfactory as did the gelatin, because a large amount of the material was required to give the same results as a much smaller quantity of gelatin.

The formula found to be the most satisfactory and the one used by the writer is as follows:

Lead nitrate	331.4 gm.
Disodium arsenate	311.96 gm.
Gelatin	17.35 gm.

The gelatin is added to the crystals of sodium arsenate and the mixture is dissolved in a small quantity of hot water and diluted to make 10 liters. The lead salt is likewise dissolved in hot water and diluted to make

[1] Accepted for publication Aug. 11, 1923.

[2] HAYWOOD, J. K., and MCDONNELL, C. C. LEAD ARSENATE. U. S. Dept. Agr. Bur. Chem. Bul. 131, p. 17. 1910.

10 liters. These are then mixed by slowly pouring the solution of lead nitrate into the solution of sodium arsenate and gelatin, stirring continuously. During this operation the mixture should be tested occasionally with potassium iodid test paper, to determine when the lead salt is slightly in excess. This will be indicated by the paper turning yellow.[3] A large excess should be avoided, for the nitrate may injure the foliage.[4]

CONCENTRATION

The physical properties of colloidal lead arsenate depend upon the concentration of the solutions of lead nitrate and sodium arsenate at the time of mixing. The following table shows the results of different concentrations upon the physical properties of the resulting arsenate:

Concentration.	Properties.
1/2 molar solution.....	Curdy precipitate, particles large; settles rapidly.
4/10 molar solution.....	Precipitate viscous and cheesy; settles rapidly.
3/10 molar solution.....	Precipitate viscous; particles seem to be large and flaky.
2/10 molar solution.....	Precipitate not curdy, particles large; settles slightly on standing.
1/10 molar solution.....	Precipitate fine and creamy; settles only slightly after standing several days.
1/100 molar solution....	Very minute particles.

It will be noted from this table that very concentrated solutions result in a curdy precipitate of lead arsenate, which is undesirable from a spraying standpoint, as it will form large, hard particles upon the foliage and will not spread evenly. Very dilute solutions tend to form small, needlelike crystals. These may be seen under the high power of a compound microscope, and the suspension of the arsenate in water has a silky, crystalline appearance. The results indicate that 1/10 molar is the most desirable concentration to use in the preparation of colloidal arsenate.

PHYSICAL PROPERTIES

The arsenate of lead prepared by the formula mentioned, using 1/10 molar solutions, is a colloid; it will pass through filter paper and remain in suspension several days. The "Brownian movement" of these small particles (1 micron or less in diameter) may be seen under high power of a compound microscope. The material, when sprayed upon the leaf, forms a very thin film over the entire surface, which adheres closely and is not easily washed off by rains.

SUMMARY

A colloidal lead arsenate may be prepared by precipitating lead arsenate in the presence of a protective colloid, such as gelatin, by the chemical action between lead nitrate and disodium arsenate.

The colloidal arsenate is composed of very fine particles which will remain in suspension for several days. When sprayed upon a leaf the material forms a thin, smooth film over the entire surface, which is not easily washed off by rains.

[3] The test paper may be prepared by soaking filtering or blotting paper in a concentrated solution of potassium iodid and allowing it to dry.

[4] To make approximately 1 pound of lead arsenate, use 14.03 ounces of disodium arsenate, 14.91 ounces of lead nitrate and 1 ounce of gelatin. The salts should be mixed and dissolved as before, except that each salt should be diluted to make 3.5 gallons. The resulting lead arsenate may be diluted to any desired strength for spraying.

ACTIVE CHLORIN AS A GERMICIDE FOR MILK AND MILK PRODUCTS[1]

By HARRISON HALE, *Head, Department of Chemistry, University of Arkansas, and* WILLIAM L. BLEECKER, *Bacteriologist, Arkansas Agricultural Experiment Station*

The use of chlorin in some form as a germicide in water purification is well established, and continued experience has demonstrated its efficiency and proven that it is not harmful.[2] Exceedingly delicate tests for an excess of free chlorin have been devised, perhaps the most satisfactory being that of Ellms and Hauser,[3] using 0.01 per cent solution of ortho-tolidin in 10 per cent hydrochloric acid. Even when those tests fail to show the presence of free chlorin in water the germicidal action continues, as shown by bacterial counts. The chlorin apparently forms complex compounds with the organic matter in the water, which, while not giving the test for free chlorin, still continues to possess germicidal properties.[3]

The object of this investigation has been to determine whether a similar use of active chlorin as a germicide for milk and milk products is possible and, if so, in what form it is best applied.

Preliminary tests made in March, 1918, on ice cream, by placing small amounts of sodium hypochlorite in the mixture of pasteurized cream before freezing, gave these results:

TABLE I.—*Preliminary tests*

Sample.	Available chlorin.	Bacteria per cc.	*B. Coli Communis*—No. of positive tests in 5 samples, 1 cc. each.	Acidity after standing 72 hours.
Control	None.	15,000	One.	1.000
Sample 1	1:16000 "BK"	12,000	None.	.909
Sample 2	1:8000	3,000	None.	.727

In making the bacteriological tests regularly the following methods were used:

The milk was weighed in sterile flasks, so that each flask contained exactly 100 gm. The chlorin in solution was then added to each flask in sufficient quantity to make the desired solution, the amount of active chlorin in each cc. having been previously determined by titration. In every case the milk in one flask was left untreated as a control. After the chlorin solution had been added the flasks of milk were allowed to stand at room temperature for the desired length of time with frequent agitation.

[1] Accepted for publication May 2, 1923. Research Paper No. 1, Journal Series, University of Arkansas.

[2] RACE, Joseph. CHLORINATION OF WATER. viii, 158 p., illus. New York and London. 1918. Bibliographies at ends of chapters.

[3] ELLMS, J. W., and HAUSER, S. J. ORTHO-TOLIDINE AS A REAGENT FOR THE COLORIMETRIC ESTIMATION OF SMALL QUANTITIES OF FREE CHLORINE. *In* Jour. Indus. and Engin. Chem., v. 5, p. 915-917, 1030. 1913.

——— ——— THE EFFECT OF FERRIC SALTS AND NITRITES ON THE ORTHO-TOLIDINE AND STARCH-IODIDE TESTS FOR FREE CHLORINE. *In* Jour. Indus. and Engin. Chem., v. 6, p. 553-554. 1914.

RACE, JOSEPH. OP. CIT. p. 82.

The milk was then plated out as follows: After thorough agitation 0.1 cc. was removed in a 0.1 cc. precision pipette and added to 100 cc. of sterile normal salt solution in a sterile flask. After thorough mixing, 1 cc. of this solution was then transferred to each of two sterile Petri dishes, sterile nutrient agar added, and the contents thoroughly mixed. This gave a 1:1000 dilution of the milk and was found to be satisfactory so far as the counting of the colonies was concerned. In a few instances it was necessary to estimate the number of colonies by counting the colonies in 12 square centimeters and multiplying the average number by the area of the plate. In doing this a Bausch and Lomb colony counter was used. In the majority of cases, however, the actual number of colonies could be determined.

In all tests the plates were kept at 37.5° C. for 48 hours before being counted. In the earlier tests fermentation tubes containing 1 per cent lactose broth were used to indicate *B. Coli*, but later the milk was plated out with Bacto-purple lactose agar. While this medium is also affected by *Streptococcus lactis*, the colonies of this organism can be differentiated readily from those of *B. Coli*.

Chlorin was first used as a solution of sodium hypochlorite, part of the time as a chemically pure solution prepared in our laboratory, part of the time as the commercial product "BK." The chlorin water used was made in the laboratory, as was some of the solution of calcium hypochlorite, though some high grade "bleach" or "chlorid of lime" was also used. All of these solutions were carefully titrated against an arsenic trioxid solution to determine the amount of active chlorin each contained at the time used.

Since the solution of "BK" was alkaline, two series of tests were made simultaneously. In one, the alkaline solution was used (Table II), and in the other the neutralized solution (Table III).

TABLE II.—*Action of sodium hypochlorite ("BK")—alkaline*

Active chlorin.	Time acting before plating.	Bacteria.	Gas in lactose broth.	Flavor of chlorin.
Untreated	1 hour	2, 160, 000	10 per cent.	
1:500	do	100	None	Yes.
1:2500	do	6, 000	do	Trace.
1:5000	do	7, 200	do	Do.
1:25000	do	10, 700	do	None.
1:50000	do	16, 500	5 per cent.	Do.

TABLE III.—*Action of sodium hypochlorite ("BK")—neutralized*

Active chlorin.	Time acting before plating.	Bacteria.	Gas in lactose broth.	Flavor of chlorin.
Untreated	1 hour	2, 160, 000	10 per cent.	
1:500	do	0	None	Trace.
1:2500	do	4, 800	do	Do.
1:5000	do	7, 250	Trace	Do.
1:25000	do	10, 000	do	None.
1:50000	do	13, 500	do	Do.

It will be noticed that the milk used in the tests was of very poor quality, as shown by the large number of organisms in the untreated milk and by the amount of gas produced in lactose broth. In both tests the results show germicidal action in proportion to the active chlorin present. Even in the 1:50000 dilution there was a marked decrease in the number of bacteria.

The next test, the results of which are given in Table IV, was practically a duplicate of the test shown in Table II, except that the milk was of much better quality and different dilutions of chlorin were used.

TABLE IV.—*Action of sodium hypochlorite ("BK")—alkaline*

Active chlorin.	Time acting before plating.	Bacteria.	Gas in lactose broth.	Flavor of chlorin.
Untreated	1 hour	63, 500	None	
1:1000	do	13, 100	do	Yes.
1:5000	do	44, 100	do	Trace.
1:10000	do	50, 000	do	Do.
1:25000	do	37, 500	do	None.
1:50000	do	37, 800	do	Do.
1:100000	do	40, 950	do	Do.
1:250000	do	50, 500	do	Do.

These results, and others to be given, show that a germicidal effect is produced even in extreme dilutions, but that it is not always definitely proportional to the amount of active chlorin present. This variation is probably due to differences in composition and in quality in the milk used.

Investigation of the best form or source of the active chlorin was then undertaken.

Authors of the ortho-tolidin test claim that one part of active chlorin in 200,000,000 parts of water can be detected by this method. This was verified, while the starch-iodid showed a sensitiveness of one part in nearly 10,000,000 parts of water. When applied to milk the ortho-tolidin test was found valueless, as no color appeared except with very high concentrations. When starch-iodid is used in milk containing active chlorin a sensitiveness of one part in 160,000 was the best that could be obtained, much lower than that necessary for a satisfactory reagent. Moreover, the color faded rapidly when only small amounts of chlorin were present.

A study was made of the rate of disappearance of active chlorin from chlorin water, sodium hypochlorite, and calcium hypochlorite. To determine the rapidity at which the active chlorin disappeared, the following tests were made at room temperature, using starch-iodid as indicator, the time stated showing the difference between the time of the addition of the active chlorin and the time a negative test was shown.

This disappearance of the active chlorin is probably due to the formation by the chlorin of compounds with the organic matter in the milk, and the chlorin in the chlorin water, being more readily available, acts more rapidly.

TABLE V.—*Study of the rate of disappearance of active chlorin from various sources*

Concentration.	Calcium hypochlorite.	Sodium hypochlorite.	Chlorin water.
1 part active chlorin in—			
100,000	13 minutes	0 to ¼ minute	0.
50,000	10 to 20 minutes	¼ to ½ minute	0.
20,000	20 to 30 minutes	½ to 1 minute	0.
10,000	3½ to 4½ hours	1 to 5 minutes	0 to 1 minute.
6,000	11 to 21 hours	5 to 15 minutes	1 to 10 minutes.
3,000	80 to 96 hours	3 to 4 hours	10 to 20 minutes.
1,000	Over 96 hours	10 to 21 hours	20 to 30 minutes.

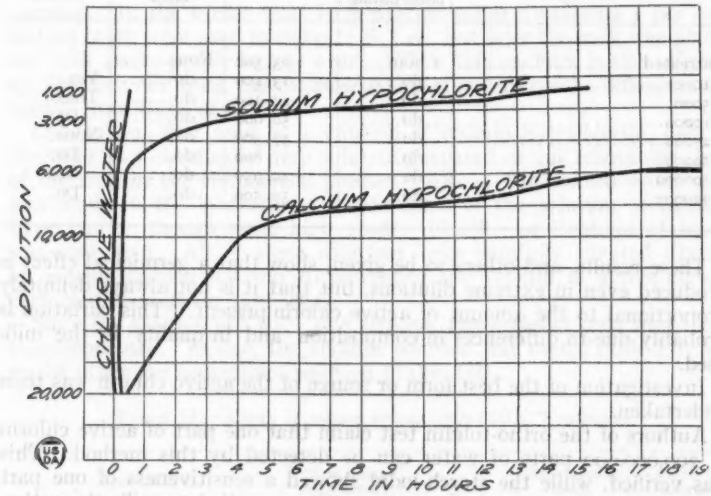


FIG. 1.—Diagram showing the relative rapidity of disappearance of active chlorin from chlorin water, sodium hypochlorite, and calcium hypochlorite, respectively.

Further bacteriological tests were also made. In Table VI the source of the chlorin was chlorin water. The milk used was of poor quality, having been inoculated with *B. coli communis* the night before, and had stood all night at room temperature. Considering the quality of the milk, a decided germicidal effect is seen.

TABLE VI.—*Action of chlorin water*

Active chlorin.	Time acting before plating.	Bacteria.	Gas in lactose broth.	Flavor of chlorin.
Untreated	1 hour	600,000	10 per cent.	
1:4000	do.	82,000	Trace	None.
1:7000	do.	100,000	do.	Do.
1:12000	do.	167,300	5 per cent.	Do.
1:15000	do.	243,000	do.	Do.
1:20000	do.	285,500	do.	Do.
1:50000	do.	443,000	10 per cent.	Do.

The most notable fact about using chlorin in this form is that no flavor is imparted to the milk in even the strongest dilution used in this test, 1:4000. A dilution of 1:3000 gave a very slight flavor, but one that would not be detected after a short time. Compared to this, a dilution of 1:10000 of sodium hypochlorite gave a decided flavor.

Tests for the purpose of comparing the germicidal action of active chlorin from various sources upon certain specific organisms were then made. In the first, milk was inoculated with *Staphylococcus pyogenes aureus* at 4.30 p. m., heated on the water bath to 37° C., and then allowed to stand at room temperature until 10 a. m. the next day. This organism was used as the colonies could be easily identified, as it is at times found in milk and is pathogenic. The results are given in Table VII.

TABLE VII.—*Action of chlorin water and calcium hypochlorite on Staphylococcus pyogenes aureus*

Active chlorin.	Time acting.	Number of bacteria with—	
		Chlorin water.	Calcium hypochlorite.
1:3000	4 hours	Little decrease	Practically no decrease.
1:5000	do	Decided decrease	Do.
1:7000	do	do	Do.
1:10000	do	Practically sterile	Do.
1:15000	do	Decided decrease	Do.
1:20000	do	do	Do.

A comparison was then made of the action on *Sarcina lutea*. This organism was selected because it gives a characteristic, easily differentiated colony, and is about as sensitive to the action of chemicals as the pathogenic bacteria usually found in milk. Milk inoculated at 4.30 p. m. was left standing at room temperature till the next morning, when it was placed in the incubator until noon before being treated. After standing nearly 20 hours it was very acid.

TABLE VIII.—*Action of chlorin water, sodium hypochlorite, and calcium hypochlorite on Sarcina lutea*

Active chlorin.	Time acting.	Number of *Sarcina lutea* per plate, with—		
		Chlorin water.	Sodium hypochlorite.	Calcium hypochlorite.
Untreated		100	100	100
1:1000	2 hours	0	0	0
1:3000	do	1	0	2
1:6000	do	10	25	0
1:10000	do	1	5	1

No total count of the colonies on these plates was made. The photographs of the plates show very clearly the effect of the chlorin from the three sources. (Pl. 1, 2, 3.)

A final comparison was made of the action upon *B. coli communis*. The milk was heavily inoculated at 4.30 p. m., practically the whole of a 24-hour culture being added to one quart. The culture was suspended

in sterile normal salt solution and then passed through filter paper to prevent the addition of organisms in large masses. After standing at room temperature till next morning it was placed in the incubator at 37° C. until 2 p. m.

In this test the plates of each dilution were run in duplicate as in the others, but in this case one plate of each dilution was plated out on Bacto-purple lactose agar. This is a medium prepared by the Digestive Ferments Co. and contains 0.0025 part dibromcresolsulphonephthalein as an indicator. When neutral this medium is a beautiful purple, but the least formation of acid turns it yellow. If but a few colonies of acid-producing organisms are present, they will appear as yellow colonies on a background of purple. An abundance of acid colonies turns the entire medium yellow.

No count was made of the colonies on these plates, there being hundreds of thousands from the untreated milk. All of the plates to which chlorin had been added showed considerable decrease in bacteria. In only two plates did the medium remain purple. In the 1:1000 dilution of chlorin water and of sodium hypochlorite all of the *B. coli* were killed, but in the 1:1000 of calcium hypochlorite and in the dilutions of 3000, 6000, and 10000 from all three sources, enough *B. coli* remained alive and viable to change the purple medium to yellow.

A series of tests involving a change in the time of action of the three sources of active chlorin was then undertaken. The milk used was inoculated with about half of a 24-hour culture of *B. coli communis* and incubated for six hours at 37° C. before the chlorin was added.

TABLE IX.—*Time factor in germicidal action of chlorin water, sodium hypochlorite, and calcium hypochlorite*

Source of chlorin.	Time acting.	Active chlorin.	Bacteria.	Bactose purple lactose agar.
	Hours.			
Chlorin water	¼	1:1000	7,000	Unchanged.
Do	¼	1:3000	160,000	Yellow.
Do	¼	1:6000	350,000	Do.
Do	¼	1:10000	630,000	Do.
		Untreated.	1,500,000	Do.
Sodium hypochlorite	1½	1:1000	140,000	Unchanged.
Do	1½	1:3000	525,000	Yellow.
Do	1½	1:6000	777,000	Do.
Do	1½	1:10000	770,000	Do.
		Untreated.	1,500,000	Do.
Calcium hypochlorate	1½	1:1000	1,000,000	Yellow.
Do	1½	1:3000	450,000	Do.
Do	1½	1:6000	577,500	Do.
Do	1½	1:10000	630,000	Do.
		Untreated.	1,500,000	Do.
Do	19	1:1000	4,000	Unchanged.
Do	19	1:3000	721,000	Yellow.
Do	19	1:6000	1,610,000	Do.
Do	19	1:10000	2,625,000	Do.
		Untreated.	10,920,000	Do.

The time factor in these tests is most noteworthy, showing an agreement with the results stated in Table V, in that chlorin water produces fully as good effects in 45 minutes as sodium hypochlorite does in 1 hour and 30 minutes and calcium hypochlorite in 19 hours.

That the germicidal action of the chlorin takes place soon after its addition is seen in the following results, sodium hypochlorite being used

TABLE X.—*Early germicidal action as shown in case of sodium hypochlorite*

Active chlorin.	Time acting before plating.	Bacteria.	Gas in lactose broth.
	Hours.		
Untreated	½	190, 000	None.
	2	200, 000	Do.
1:3000	½	12, 500	Do.
	2	30, 500	Do.
1:5000	½	20, 000	Do.
	2	25, 000	Do.
1:10000	½	32, 500	Do.
	2	30, 000	Do.
1:25000	½	42, 500	Do.
	2	25, 000	Do.
1:50000	½	175, 000	Do.
	2	80, 000	Do.
1:100000	½	200, 000	Do.
	2	212, 000	Do.

The results in Table XI may be compared with those given in Table IX.

TABLE XI.—*Time factor in germicidal action of chlorin water, sodium hypochlorite, and calcium hypochlorite*

Source of chlorin.	Time acting.	Active chlorin.	Bacteria.	Bactose purple lactose agar.
	Hours.			
Chlorin water	2	1:1000	60, 000	Unchanged.
Do	2	1:3000	190, 000	Yellow.
Do	2	1:6000	110, 000	Do.
Do	2	1:10000	200, 000	Do.
		Untreated.	492, 000	Do.
Sodium hypochlorite	2	1:1000	Sterile.	Unchanged.
Do	2	1:3000	85, 000	Yellow.
Do	2	1:6000	140, 000	Do.
Do	2	1:10000	252, 000	Do.
		Untreated.	492, 000	Do.
Calcium hypochlorite	2	1:1000	Sterile.	Unchanged.
Do	2	1:3000	420, 000	Yellow.
Do	2	1:6000	301, 000	Do.
Do	2	1:10000	392, 000	Do.
Do	2	Untreated.	492, 000	Do.

SUMMARY

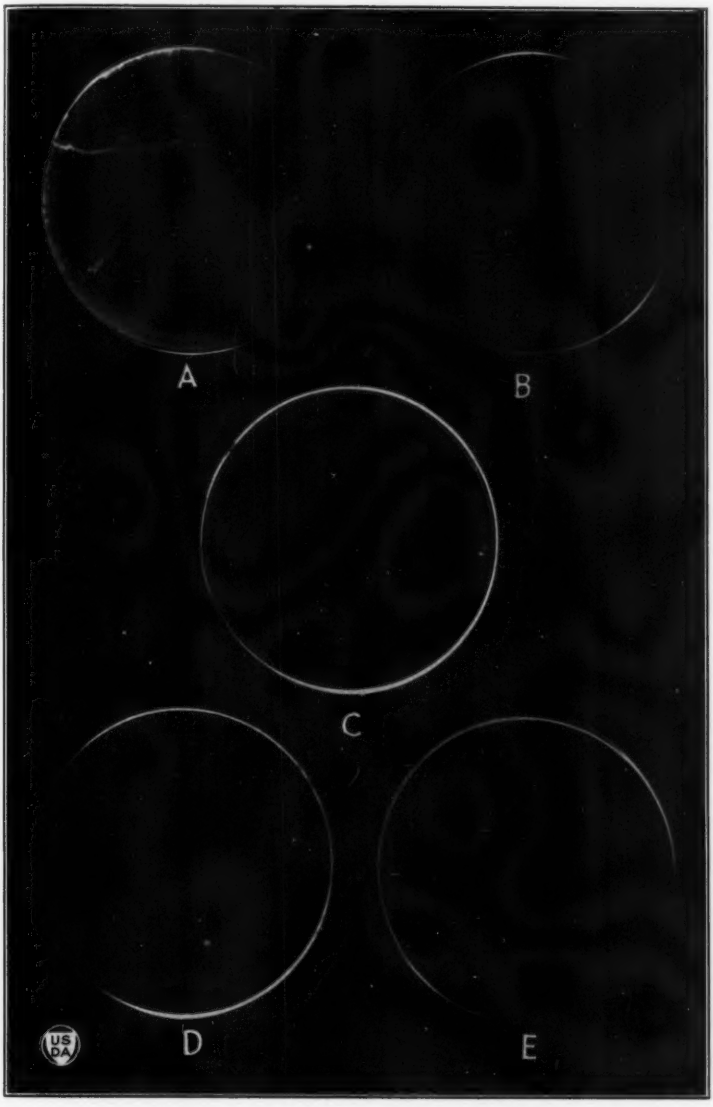
1. Active chlorin does act as a germicide in milk and in ice cream with a reduction in the number of bacteria in general proportional to the amount of active chlorin present.

2. Chlorin water gives as satisfactory results in 45 minutes as sodium hypochlorite does in 90 minutes or calcium hypochlorite does in 19 hours. The chlorin water could be used in higher concentrations than the other two without an effect upon the flavor.

It is not the wish of the authors that this paper be considered in any way as a recommendation of chlorin for treating market milk. Much more work must be done before any final decision can be reached. We hope our results may stimulate further research.

PLATE 1

Plates showing action of chlorin water, involving various concentrations of chlorin, on milk inoculated with Sarcina lutea. A, untreated milk; B, active chlorin 1:1000; C, active chlorin 1:3000; D, active chlorin 1:6000; E, active chlorin 1:10000.


A
B
C
D
E
USDA

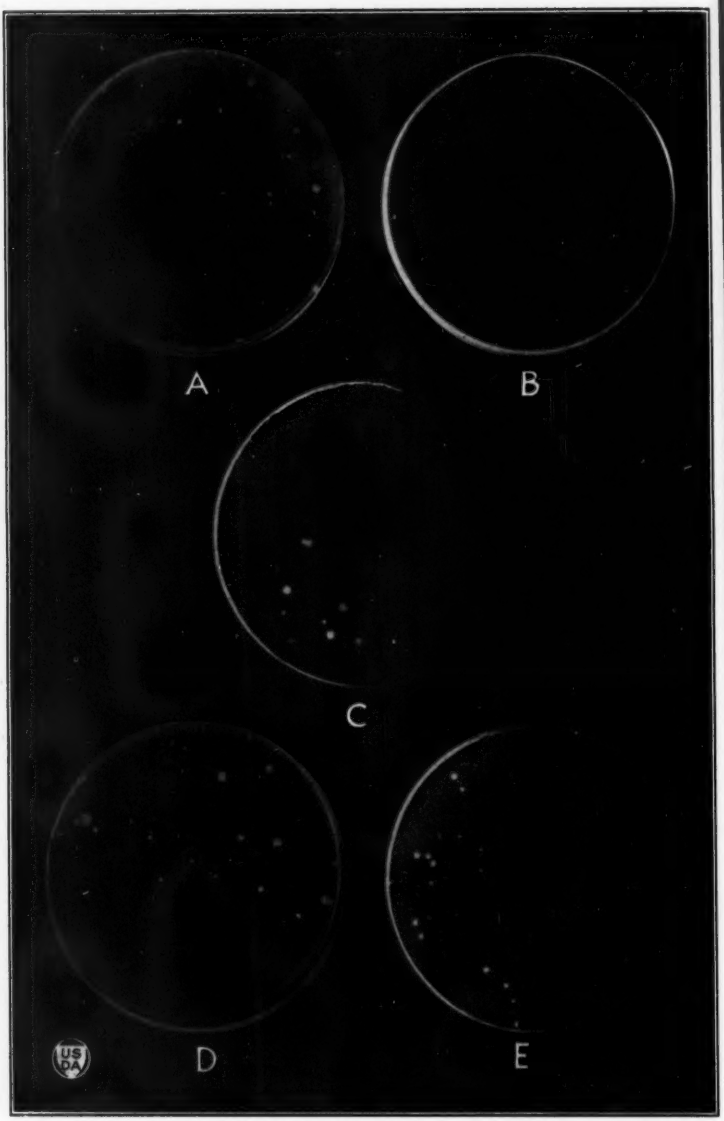
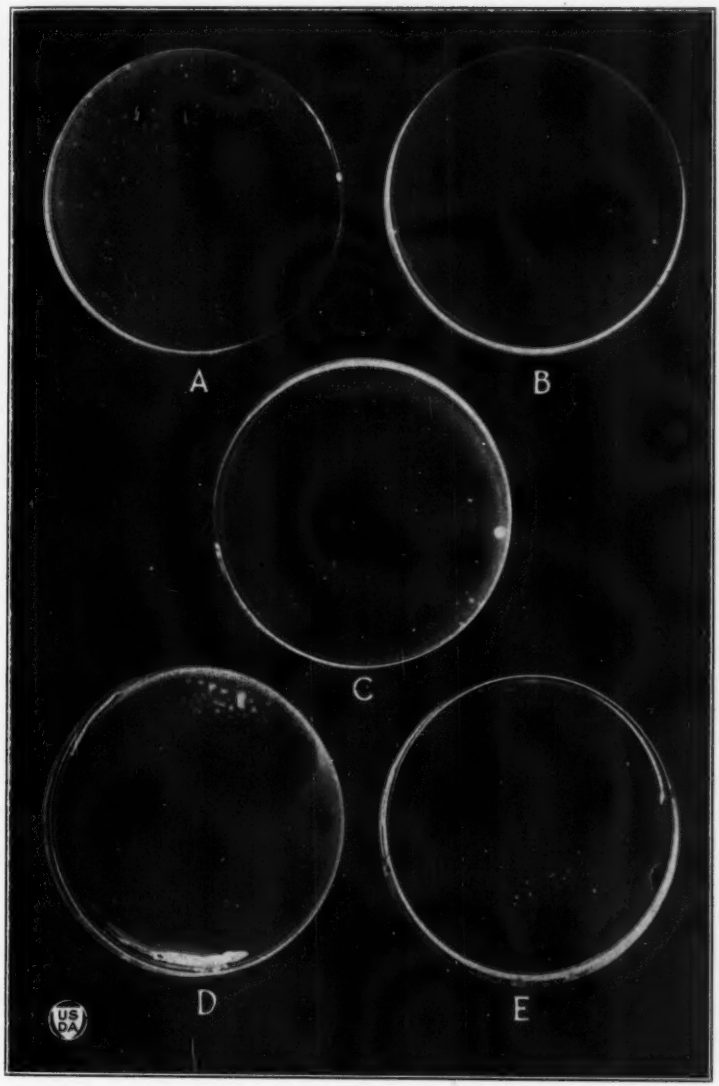

A
B
C
D
E
USDA

PLATE 2

Plates showing action of sodium hypochlorite, involving various concentrations of chlorin, on milk inoculated with Sarcina lutea. A, untreated milk; B, active chlorin 1:1000; C, active chlorin 1:3000; D, active chlorin 1:6000; E, active chlorin 1:10000.

PLATE 3

Plates showing action of calcium hypochlorite, involving various concentrations of chlorin, on milk inoculated with Sarcina lutea. A, untreated milk; B, active chlorin 1:1000; C, active chlorin 1:3000; D, active chlorin 1:6000; E, active chlorin 1:10000.


A
B
C
D
E
USDA